LYMPHOCYTE HETEROGENEITY IN TELEOSTS AND REPTILES

By

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KEY TO ABBREVIATIONS

Con A.....concanavalin A

CPM.....counts per minute

DNA.....deoxyribonucleic acid

G-MEM.....alligator mimimum essential medium

H chain.....heavy chain

^{3}H.....tritium

Ig.....immunoglobulin

L chain.....light chain

LPS.....lipopolysaccharide

MEM.....mimimum essential medium

MLC.....mixed lymphocyte culture

PFC.....plaque-forming cell

PHA.....phytohemagglutinin

PPD.....purified protein derivative

PWM.....pokeweed mitogen

Ra-BIg.....rabbit anti-bluegill immunoglobulin

Ra-GL.....rabbit anti-grouper light chain

Ra-M IgM.....rabbit anti-mouse IgM

RBC.....red blood cell

RPMI 1640.....Roswell Park Memorial Institute medium 1640

SDS.....sodium dodecyl sulfate

SRBC.....sheep red blood cell

TCA.....trichloroacetic acid

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The purpose of this research was to study the characteristics of the lymphoid cells from two ectotherms, the bluegill, a representative teleost, and the Florida alligator, a representative reptile. The questions approached were 1) whether or not these animals possessed a heterogeneity of lymphocytes akin to T- and B-cells of higher animals, 2) whether a cellular basis for the effects of temperature on the immune response of ectotherms could be obtained, and 3) whether there are membrane-associated immunoglobulins in fish.

Hypaque-Ficoll centrifugation was used as a separation technique for the isolation of lymphocytes. In vitro mitogenic studies of isolated lymphocytes from each species established that homologous serum was the most satisfactory medium supplement. Bluegill studies demonstrated that the health or physiological state of laboratory maintained fish appeared to be important in obtaining low background levels of DNA synthesis. Variables found to be important in alligator lymphocyte studies were the NaCl concentration in the medium and the age of the serum donor.

Studies of the bluegill have shown that there are at least two subpopulations of lymphocytes. One population was stimulated by PHA (and Con A) at 32°C and very poorly at 22°C and was depleted by antibrain plus complement treatment. The other population is LPS responsive at both 32°C and 22°C, although responsiveness at 22°C was always greater and was depleted by removal of rabbit RBC rosetted lymphocytes from the total population.

Temperature was also shown to be an important factor in *in vitro* antigenic stimulations. *In vitro* SRBC primed cultures maintained at 32°C elicited a very good plaque-forming cell response to SRBC's whereas 22°C maintained cells gave no response. The temperature effects on the *in vitro* cultures are discussed in reference to the reported *in vivo* temperature effects on the teleost immune functions.

Evidence has been presented which argues for the presence of at least two cell populations of lymphocytes in the alligator. Summarized these are 1) differences in the magnitude of stimulation with the different mitogens, 2) differences in the combined effects of the mitogens, 3) a significant increase in immunoglobulin producing cells in LPS-stimulated cultures, 4) populations of cells adherent or nonadherent to glass wool with different responses to LPS and PHA, 5) the depletion of responsiveness to LPS by cytotoxic treatment with an anti-immunoglobulin plus complement without reducing the responsiveness to PHA, and 6) the depletion of responsiveness to LPS by removing immunoglobulin bearing cells.

Environmental temperature was shown to effect the *in vitro* mitogenic responses of cultured alligator lymphocytes. Although there were some

fluctuations in PHA responsiveness, LPS responses dropped significantly during the winter months or when alligators were housed at 16°C.

T-like and B-like designations were assigned to the different populations in the bluegill and alligator based on arguments by analogy to bird and mammalian T- and B-lymphocyte characteristics.

Studies of membrane immunoglobulins on bluegill lymphocytes from blood, anterior kidney, spleen, and thymus revealed that over 90% of the lymphocytes exhibited membrane immunoglobulin determinants as revealed by immunofluorescence. The majority of these cells were observed to undergo patching and capping when the membrane proteins were complexed with antisera to fish immunoglobulins. Lactoperoxidase catalyed radioiodination, detergent lysis and immunoprecipitation with appropriate anitsera were employed to study the properties of this membrane immunoglobulin. Quantitation indicated the average amount of immunoglobulin determinants for bluegill lymphocytes to be similar to that present on mouse B-cells. Physicochemical characterization of labeled membrane immunoglobulin from bluegill lymphocytes suggested that only one class of immunoglobulin heavy chain was present and that about one-half of this material resembled the monomeric IgM-like proteins present in bluegill serum.

CHAPTER I
INTRODUCTION

Immunity in the vertebrates may be defined as a response of an animal to a foreign substance (an antigen or immunogen) introduced into its body. The response is specific in that it is directed only to the antigen introduced and is characteristically more pronounced and occurs sooner if the same antigen is reintroduced at a later time (43).

Immune responses in birds and mammals may be cellular (specifically reactive cells) or humoral (antibody mediated) (43,54,93). Characteristic cellular responses are delayed type hypersensitivity reactions, graft rejection and graft-versus-host reactions. Specific cellular responses are transferable by lymphocytes. Humoral responses are characterized by the production of antibody directed to an antigen and the resulting immunity is transferable by serum.

The lymphocyte has been demonstrated to be the principal cell type involved in the immune responses of birds and mammals. Although lymphocytes are morphologically identical, two major subpopulations have been identified based on ontological origin and functional analysis (43,54, 93). One subpopulation, the T-(thymus derived) lymphocyte, is the functional cell in cellular mediated responses. The other subpopulation, the B-(bursa derived in birds or bursal equivalent in mammals) lymphocyte, is the functional cell in producing antibodies in humoral responses. T- and B-lymphocytes have been further characterized on the basis of cell surface determinants and *in vitro* responses to mitogens, antigens and

mixed lymphocyte reactions (43,53,54,93). B-cells respond to different mitogens (e.g lipopolysaccharide), have demonstrable levels of immunoglobulin on their surfaces, and are stimulated by mitogens or antigens to synthesize immunoglobulin or antibody. In contrast T-cells proliferate in response to different mitogens (e.g. phytchemagglutinin and concanavalin A), do not have surface immunoglobulin (or at least easily demonstrable levels), express surface differentiation antigens not found on B-cells (e.g. Thy-1) and are the responding cells in mixed lymphocyte reactions.

A unique feature of the lymphocytes involved in the immune response is the requirement of T-B cell cooperation in most responses leading to antibody production, even though the T-cell does not make antibody (49, 52,54,81). This requirement is best illustrated using hapten-carrier antigen complexes (19,28,54,69) in which carrier recognition by T-cells is required before B-cells can make antibody to the hapten. T-cells are also involved in the control of the 19S to 7S switch (IgM to IgG antibodies) as well as maturation of the humoral response (increase in antibody affinity with time after immunization) (19,54,69). It should be pointed out that except for a few antigens which are structurally very repetitious (the T-independent antigens capable of reacting directly with B-cells), both cell types must interact to elicit a response to most antigens (T-dependent antigens) (28,54,69,82).

The majority of much of the research on the immune systems briefly described above has been in birds and mammals, both of which are endothermic. With the exception of limited studies of the amphibians, studies of the cellular basis of the immune systems in ectotherms have not been done. Although one could postulate the existence of T-like and

B-like cells on the basis of graft rejection and antibody production (discussed in detail later) direct evidence of lymphocyte heterogeneity in any ectotherm has not been obtained. Furthermore the molecular or cellular bases for the commonly observed effects of environmental temperature on the immune responses of numerous ectotherms (7,37) have not been investigated.

The purpose of the research undertaken here was to study the characteristics of the lymphoid cells from two ectotherms, the bluegill, a representative teleost, and the Florida alligator, a representative reptile. The major questions approached were 1) whether these two animals possessed classes of lymphocytes akin to T- and B-cells of higher animals, 2) whether a cellular basis for the effects of temperature on the immune response of ectotherms could be obtained, and 3) whether there are membrane associated immunoglobulins in fish.

CHAPTER II
LYMPHOCYTE HETEROGENEITY IN THE BLUEGILL

Introduction

Considerable evidence from in vivo studies indicates that teleost fish can mount a diversity of immune responses. Teleosts are capable of responding to a wide variety of antigens with both primary and secondary responses (4,7,15,32,37,51,112) with the only apparent major difference from mammals being that there is no discernable "IgM → IgG switch" in the fish (1, 16,27,62,111, 121). In fact the evidence available to date shows that many species of fish synthesize only 16S tetrameric IgM-like immunoglobulin (1,16,27,62,111). In those fish also possessing low molecular weight serum immunoglobulins, the 7S molecules appear to resemble monomeric forms of the tetramer and hence it seems that fish are lacking an IgG equivalent (21,29,30,31,55,75). Attempts to demonstrate IgA-or IgE-like molecules or activities in fish have also been unsuccessful (31). Thus, in light of these latter deficiencies it would seem appropriate to speculate that while fish possess cells of B-like function, their number of immunoglobulin classes is somewhat limited. Several investigators have also demonstrated the ability of fish to reject both first and second set scale transplants with the second set rejections occurring more rapidly (12,59,60,61,88). Thus, again arguing by analogy, it appears that fish have cells with T-like function. Furthermore, studies on three different species of fish have revealed the

existence of the so-called hapten-carrier effect (46,106,121). Since this "helper effect" is considered to result from T-B cell collaboration in mammals it appears that fish may also have this coordinated function in their immune response system.

Since fish are ectothermic animals it is not surprising that numerous reports of temperature influences on immune responses have appeared. The classic studies of Bisset (10), Cushing (40) and Hildemann and Cooper (61) demonstrated that temperature can have a profound role in these responses. The more recent studies of Avtalion have served as a basis for beginning to understand the mechanism of these effects (7). He has shown that humoral responses in the carp are a two-step process: 1) a temperature-sensitive step requiring relatively high temperatures for antigen recognition and 2) a temperature-insensitive step which results in antibody production. Furthermore, Avtalion suggests that there are at least three cell types involved; 1) X cells (T-like) which are sensitive to low temperatures and are involved in priming and tolerance and 2) Y and Z cells (B-like) which are involved in memory and antibody formation respectively. It must be pointed out however that direct proof for the existence of multiple types of immunocompetent cells in fish is lacking.

More recently Etlinger et al. (46) presented evidence that rainbow trout have two lymphoid cell types. This evidence is based on responses of leukocytes isolated from various lymphoid organs to the mammalian T and B cell mitogens. Thymocytes responded only to Con A (a T-cell mitogen in mice and man) and anterior kidney leukocytes responded only to LPS or PPD (B-cell mitogens). The unique pattern of tissue localization of cells responsive to mammalian T- and B-lymphocyte mitogens was taken as evidence for lymphocyte heterogeneity in rainbow trout.

Smith et al. (102), Chiller et al. (26), and Pontius and Ambrosius (89) have studied the cellular responses of teleosts to sheep red blood cells and have demonstrated antibody-forming cells in the spleen and anterior kidney. Further studies by Sailendri and Muthukkaruppan have shown an appreciable number of antibody-forming cells in the thymus as well (96,97). One could thus conclude that fish have a B-cell equivalent, as defined by the ability of plasma-like cells to produce antibody. However, the presence of antibody-forming cells in the fish thymus indicates that the thymus may not be populated with only T-like cells as Etlinger's work suggests. Only in experimentally induced circumstances are antibody-producing cells (B-cells) found in mammalian thymuses (37). In addition, > 90% of the cells isolated from thymuses of four different species of fish have demonstrable levels of immunoglobulin on their surfaces (44,45,46,116). Although there is some controversy as to whether or not mammalian T-cells have surface immunoglobulins (to be discussed further in Chapter IV), the consensus is that if T-cells do have surface immunoglobulins they are present in very small amounts and only B-cells have readily demonstrable levels of surface immunoglobulin. Therefore, in light of the existing data, there is some question as to whether fish thymocytes are similar to mammalian thymocytes.

It should be pointed out that much of the data supporting the concept of two cell types (presumed to be lymphocytes) involved in immune responses in fish are only inferential and alternative interpretations may be presented. Indeed the unusual properties of the fish thymus (surface immunoglobulin expression and the presence of antibody producing cells), as well as a lack of maturation in antibody responses (34)

and the presence of demonstrable hapten-carrier effects without a 16S → 7S switch suggest that if a T-like cell in fish exists it may differ functionally from higher vertebrate T-cells. Summarizing the current literature, it appears that direct evidence for two lymphocyte subpopulations in fish is lacking.

The purpose of this portion of the research was to determine in a direct way if a teleost, the bluegill, has a heterogeneous population of lymphocytes akin to T- and B-cells in birds and mammals. The approach taken was three fold: 1) to define a separation technique for the isolation of relatively pure lymphocytes and to establish appropriate *in vitro* culture conditions, 2) to determine if mitogenic responses and cell surface determinants employed as T- and B-cell probes in birds and mammals are applicable to bluegill lymphocytes as *in vitro* markers, and 3) to separate differing subpopulations of lymphocytes on the basis of differences in mitogenic and cell membrane antigens. Special emphasis was placed on studying the effects of temperature on bluegill lymphocytes to determine if a cellular basis for the *in vivo* temperature effects on the immune responses in fish exist.

Materials And Methods

Experimental Animals

Bluegill (Lepomis macrochirus), a freshwater teleost, was used exclusively as a source of lymphocytes in these studies. Sexually mature male and female specimens, weighing 200-500 g, were obtained from the University of Florida's Lake Alice using cane poles, barbless hooks and bread as bait. Fish were handled with rubber gloves and kept in aerated holding tanks until transported to laboratory aquaria. One hundred twenty-five liter Nalgene tanks filled with dechlorinated water were used to maintain specimens in the laboratory. A maximum of eight fish per tank were maintained with continuous aeration and a complete change of water every 3-4 days. Fish were fed to satiation 2-3 times each week with TetraMin (Tetra Werke, West Germany). As discussed later, these holding conditions were less than ideal.

Culture Media

Roswell Park Memorial Institute (RPMI) 1640 was used as a wash medium and as a supportive medium for in vitro mitogenic studies. The complete medium used was prepared by dissolving RPMI 1640 instant tissue culture powder (Grand Island Biological Company [GIBCO], Grand Island, N.Y.), penicillin (GIBCO; 50 U/ml), streptomycin (GIBCO; 50 ncg/ml), gentamycin (Schering, Kenilworth, N.J.; 20 mcg/ml), heparin (Sigma, St. Louis, Mo.; sodium salt, 5 U/ml) and sodium bicarbonate (Mallinckrodt,

St. Louis, Mo.; 2.2 g/L) in triple-distilled water. The pH was adjusted to 7.2 with NaOH or HCl, and the solution sterilized by passage through 0.45 μ detergent free Swinex-25 millipore filters (Millipore, Bedford, Mass.).

For in vitro studies of primary immune responses (Mishell-Dutton type cultures [83]) a medium modified from Click et al. (35) was used. Modifications of the original technique included exclusion of NaOH and 2-mercaptoethanol, substitution of RPMI 1640 for Hank's and the addition of gentamycin (20 mcg/ml), heparin (5 U/ml) and sodium bicarbonate (2.2 g/L, dissolved in the initial media preparation). Concentrations of the amino acids (GIBCO), nucleic acid precusors (GIBCO), pyruvate (GIBCO), glutamine (GIBCO), vitamins (GIBCO), penicillin and streptomycin were added as described by Click et al. (35). The medium was prepared by dissolving the above ingredients in triple-distilled water, adjusting the volume and pH and sterilizing as for the preparation of RPMI 1640 (described above).

Medium Supplements

Serum and plasma sources which were tested as medium supplements for in vitro studies were fetal calf serum (GIBCO; Lot # A030113; International Scientific Ind., Inc., Cary, Ill.; Lot # 7066411), Calf Serum (GIBCO; Lot # Ro266T), human serum pools (five pools furnished by Dr. R. Waldman, University of Florida, > 50 normal human sera per pool), rabbit serum pools (New Zealand White rabbits, two pools, > 10 normal rabbit sera per pool), alligator (Alligator mississippensis) serum (Silver Springs Reptile Institute, Silver Springs, Fla.; four individual normal alligator sera), fresh water catfish (Ictaluru cerracanthus) plasma (heparinized, pool from ten catfish), large mouth bass (Micropterus

punctulatus) plasma (five heparinized pools, five normal bass per pool), giant grouper (Epinephelus itaira) serum (pool from five grouper) and bream (a collective term for all Lepomis species) plasma (heparinized pools, > 10 fish per pool). All sera or plasma were heat inactivated for 30 min at 56°C and were sterilized by Millipore filtration (0.45 μ)

Preparation of Cell Suspensions and Counting Technique

The sources of cells studied from the bluegill were blood, anterior kidney (pronephrus or head kidney), thymus and spleen (6,48,68,96,97,102, 117). Heparinized blood, obtained from the caudal vein (108) and all organs were removed aspectically. Organs were placed in sterile petri dishes containing cold RPMI 1640. A single cell suspension of each organ was prepared by gently teasing apart the organ with forceps and pipeting the teased suspension over a 60-80 mesh steel screen to remove clumps and connective tissue.

A Hypaque-Ficoll method, adapted from Boyum's Isopaque-Ficoll technique (14), was used to isolate lymphocyte populations from organ cell suspensions or heparinized blood. Hypaque-Ficoll solutions were prepared by mixing 10 parts of 33.9% Hypaque (Winthrop Laboratories, New York, N.Y.) with 24 parts or 9% Ficoll (Pharmacia, Piscataway, N.J.), Densities of prepared solutions were 1.077 ± 0.0005 g/ml (room temperature) as determined by picnometer difference weighings.

A maximum of five ml of a teased organ cell suspension or heparinized whole blood diluted 1:4 with RPMI 1640 was gently layered onto five ml of Hypaque-Ficoll in a 15 ml tube (Falcon, Oxnard, Cal.; 17 x 100 mm). Tubes were spun at room temperature in a table-top centrifuge (International Centrifuge, Boston, Mass.) for 20 min at 1000 RPM. The interface

band of cells between the Hypaque-Ficoll and the overlaying suspension medium was removed using a Pasteur pipet and diluted in cold RPMI 1640. The suspension was spun for 10 min at 1000 RPM in a refrigerated centrifuge and the cell pellet washed three times with cold RMPI 1640.

The number of phagocytic cells was assessed using collodial carbon uptake. India ink was diluted 1:10 with saline, centrifuged for 30 min at 3500 RPM and millipore filtered (0.45 μ) prior to use. One drop was added to approximately three ml of a cell suspension and the mixture incubated for 30 min at 37°C. The cells were then washed three times and May-Grunwald-Giemsa stained cytocentrifuged (Shandon-Elliott Inc., Sewickley, Penn.) mounts prepared for quantitation.

Cell counts (109) and viability (13) were determined by diluting an aliquot of the washed cell suspension in a white blood cell diluting pipet (Scientific Products, Ocala, Fla.) with 0.1% trypan blue in RPMI 1640 and counting with a Neubaurer hemacytometer (Scientific Products).

Culture Techniques

A laminar flow hood (Abbott Laboratories, Chicago, Ill.) was used as a sterile environment for all cell culture work.

A microculture method (58,107) was adapted for mitogenic stimulation and mixed lymphocyte culture assays. For mitogen studies, washed and pelleted cells were resuspended in serum or plasma supplemented RPMI 1640 and were dispensed into microculture trays (Linbro, Hamden, Conn.; U-shaped wells) at a cell concentration of 5×10^5 cells/0.2 ml/well. The mitogens used consisted of lippolysaccharide (DIFCO Labs, Detroit, Mich.) from S. typhimurium which was boiled one hr after reconstitution with triple distilled water, phytohemagglutinin P (DIFCO) and concanavalin A (Miles Labs, Inc., Kankakee, Ind.; 3x crystallized). Stock solutions

were diluted with RPMI 1640 without serum or plasma supplements and were added to appropriate wells in 20 µl volumes immediately after the cells were dispensed. Twenty microliters of RPMI 1640 without supplement or mitogen was added to control unstimulated wells.

Two-way mixed lymphocyte cultures of cells from two bluegills were prepared by adding 2.5×10^5 cells suspended in 0.1 ml of supplemented RPMI 1640 from each cell preparation (total cell concentration per well was 5×10^5/0.2 ml). Five hundred thousand cells/0.2 ml/well from each source served as controls.

Tritiated-thymidine (Schwarz-Mann, Orangeburg, N.Y.; sterile acqueous solution, pH 7.4, 1.9 Ci/mM, 1.0 mCi/ml), diluted in supplement free RPMI 1640, was added to each culture well at a concentration of 0.5 µCi/10 µl/well at 24 hr prior to harvesting.

Microculture trays were maintained in 5% CO_2 - 95% air, saturated-humidity incubators at the temperatures indicated. CO_2 content was routinely measured with a Fryrite CO_2 tester (Bacharach Instrument Company, Pittsburgh, Penn.).

Cells, mitogens and ^{3}H-thymidine were dispensed in microculture trays using 0.5, 1.0, 5 or 10 ml gas tight syringes (Hamilton, Reno, Nev.) attached to repeating dispensers (Hamilton) delivering one-fiftieth of the attached syringe volume.

For in vitro studies of primary immune responses, single cell suspensions were prepared from pooled anterior kidney, spleen, and thymus by teasing apart the organs in RPMI 1640 and seiving through a 60-80 mesh screen. The cell suspension was centrifuged and the pellet washed three times. The final cell pellet was resuspended in enriched RPMI 1640 medium (described above) supplemented with 7% bass plasma.

White, red, and dead cells were enumerated and 1 x 10^7 viable white cells in three ml of supplemented medium were aliquoted in Falcon 35 x 10 mm tissue culture dishes (Scientific Products).

Sheep red blood cells (SRBC's) used for immunization of the dissociated organ suspensions were obtained from a single sheep (Colorado Serum Comp., Denver, Col.; Sheep # 20, H type antigen). SRBC's were washed three times with RPMI 1640 and the final pellet suspended in the enriched RPMI 1640 (without supplement) to 1% of the packed cell volume. Cultures to be immunized received 0.1 ml of the 1% SRBC suspension. Controls received 0.1 ml of enriched RPMI 1640.

Culture dishes were maintained in 5% CO_2 - 95% air humidified environments as described above.

Assay for ^{3}H-thymidine Incorporation into DNA

An automatic cell harvester (Otto Hiller Company, Madison, Wis.) was used to obtain trichloroacetic acid (TCA) precipitable nucleic acid material from individual wells of cultured cells. Twenty-four hour pulsed cells were syphoned from the wells onto a glass fiber filter (Reeve Angel, Whatman, Inc., Clifton, N.J.), rinsed with saline, precipitated with 10% TCA and methanol dried. Discs, representing individually harvested wells, were punched out of the filter strip and assayed for ^{3}H using liquid scintillation counting. The scintillation cocktail used consisted of PPO (Packard, Chicago, Ill.; 16.5 g), POPOP (Packard; 0.3 g), Triton X-100 (Packard; 1.0 L), and toluene (Mallinckrodt, St. Louis, Mo.; 2.0 L). Samples were counted in mini-vials (Rochester Scientific, Rochester, N.Y.) using an automatic liquid scintillation counter (Beckman Instruments, Fullerton, Cal.; Model LS-100).

Stimulation Indices and Statistical Analysis

Means and standard deviations were determined for each data group. An F-test was used for variance analysis. The Student-t test was used to determine the statistical significance of increases over control values (20,103). A 95% or greater confidence level ($p \leq 0.05$) was used for both the F-test and the t-test.

Stimulation indices were used to express increases of mitogen stimulated cultures over control cultures or mixed lymphocyte cultures (MLC's) over controls. Indices for mitogenic studies were determined using the following equation: $\frac{\text{Mean CPM of stimulated cultures}}{\text{Mean CPM of control cultures}}$. Indices for MLC studies were calculated by using the following formula:

$$\frac{\text{Mean CPM of MLC between Fish A and Fish B}}{(\text{Mean CPM of Fish A Control Culture} + \text{Mean CPM of Fish B Control}) \div 2}.$$

Histological and Morphological Techniques

Serial cross sections of paraffin embedded gill regions of bluegill were kindly prepared by Mr. Melvin Laite (Department of Pathology, J. Hillis Miller Health Center, Gainesville, Fla.). Sectioned tissues were stained with hematoxylin and eosin.

Cell suspension smears or cytocentrifuge (Shandon-Elliot Inc.) preparations were stained with May-Grunwald-Giemsa for morphological examination.

Autoradiography

Cultured cells, pulsed with ^{3}H-thymidine for 24 hr, were pipeted from microculture tray wells, washed three times with RPMI 1640 and cytocentrifuged. Cytocentrifuged preparations were coated with nuclear track emulsion (Kodak, Inc., Rochester, N.Y.; type NTB3), exposed,

developed and fixed as described by Gormus et al (52). All processed slides were stained with toluidine blue in order to enhance microscopic examination of the cells.

Preparation of Rabbit Antisera

A rabbit anti-bluegill brain antiserum was prepared by the procedure described by Golub for mouse brain (50). Five brains were homogenized, using a tissue grinder, diluted 1:2 with PBS and 0.5 ml aliquots were emulsified with an equal volume of complete Freund's adjuvant (DIFCO) for each immunization. Sera obtained from the rabbits before immunization were used as normal rabbit serum controls. The rabbit antiserum used was obtained from one surviving rabbit which was reimmunized six times over a three-month period.

The preparation of rabbit anti-bluegill immunoglobulin is described in Chapter IV.

Cytotoxicity Assay

Complement mediated cytotoxicity of preimmune or immune normal rabbit serum and rabbit anti-bluegill brain serum on bluegill lymphocytes was accomplished by incubating 1×10^7 cells with 1:5 dilutions of rabbit sera plus a 1:10 dilution of guinea pig complement (GIBCO, lyophilized). After 1.5 hr at room temperature the cells were washed three times with RPMI 1640 and cell counts and viability determined.

Rosetting Techniques

The method of Jondal et al. (66) was followed to assess the number of lymphocytes capable of rosetting with red blood cells (RBC's) from various animals. Fresh heparinized whole blood obtained from human, sheep, rabbit, chicken, horse, ferret, guinea pig, mouse, alligator, and

bluegill were washed four times with RPMI 1640, and the white buffy coat was removed after each centrifugation. Winthrop hematocrit tubes were used to determine percentages of RBC's in each suspension and dilutions were made accordingly. Controls with only the test RBC's were routinely assayed to determine the number of white cells contributed by the RBC suspension. As a negative control, homologous RBC's were tested with bluegill lymphocytes.

Hypaque-Ficoll (ρ = 1.077) centrifugation was used to deplete rosetted lymphocytes from non-rosetted lymphocytes (41,98). Hypaque-Ficoll recovered non-rosetting cells were diluted into RPMI 1640, pelleted and washed three times.

Immunofluorescence

Immunofluorescent reagents and techniques are described in Chapter IV.

Hemolytic Plaque Assay

Cells were harvested from tissue culture dishes by gently scraping the bottom of the culture dish with a rubber policeman and pipetting the cell suspension into a conical centrifuge tube. The plate was rinsed once with 3 ml RPMI 1640 and the wash medium was added to the cell suspension. Cells were pelleted and resuspended in RPMI 1640. Viability and cell recoveries were determined prior to assaying for plaque-forming cells (PFC's).

PFC's (cells producing antibody to SRBC's) were enumerated using a slide modification (83) of the Jerne hemolytic plaque assay (65). Slides were incubated with fresh sucker fish plasma (a plasma pool from several different species of the Catostomidae family native to the Swanee

and Santa Fe Rivers in Florida) diluted 1:20 in RPMI 1640 for 3-5 hr in a 32°C, 5% CO_2 - 95% air incubator. Plaques were routinely examined microscopically prior to counting on a Quebec colony counter (New Brunswick Scientific Co., New Brunswick, N.J.).

Results

Lymphoid Organs of the Bluegill

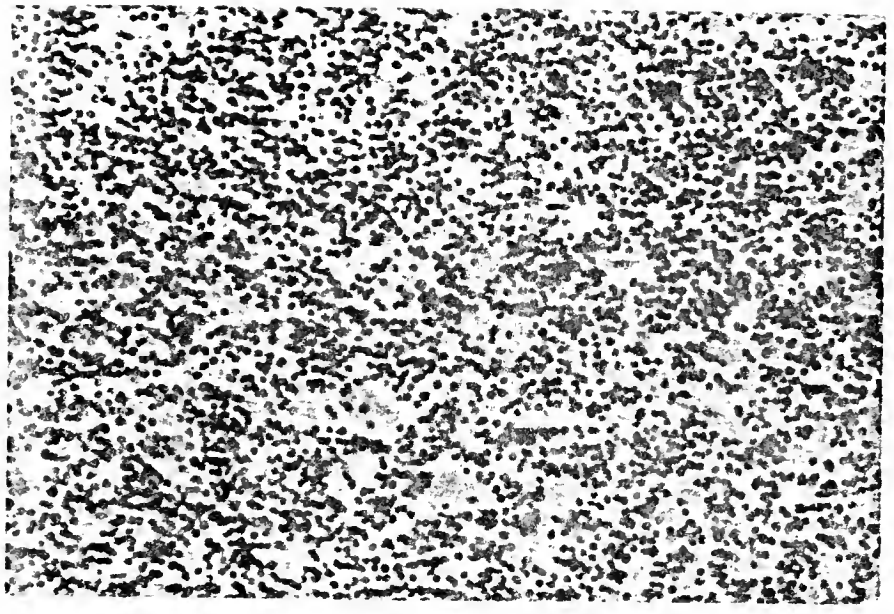
To determine which organis of the bluegill contained lymphoid cells, smears of blood or organ cell suspensions were stained with May-Grunwald-Giemsa and examined for the cell types present. Of the tissues examined, anterior kidney (head kidney or pronephros), spleen, thymus, and blood were the major sources of lymphocytes. Very few lymphocytes were found in the liver, pancreas, gonads, or posterior kidneys. Gut-associated lymphoid tissue or lymph nodes were not found.

Due to the close proximity of the thymus to the anterior kidney, serial sections were made through the gill region of a small fish ($\sim$ 100 g, < 1 yr old) and examined histologically. Figure 1 presents photomicrographs of representative sections through this region. The anterior kidney was seen to be a relatively diffuse organ containing an abundant number of blood sinuses, had a relatively large number of red blood cells and contained a heterogeneous mixture of white cells. In contrast, the thymus contained fewer red blood cells, had few white cells other than lymphocytes and contained Hassall's corpuscles. Therefore based upon both the anatomic location and the histologic characteristics, it was felt that these tissues were in fact anterior kidney and thymus.

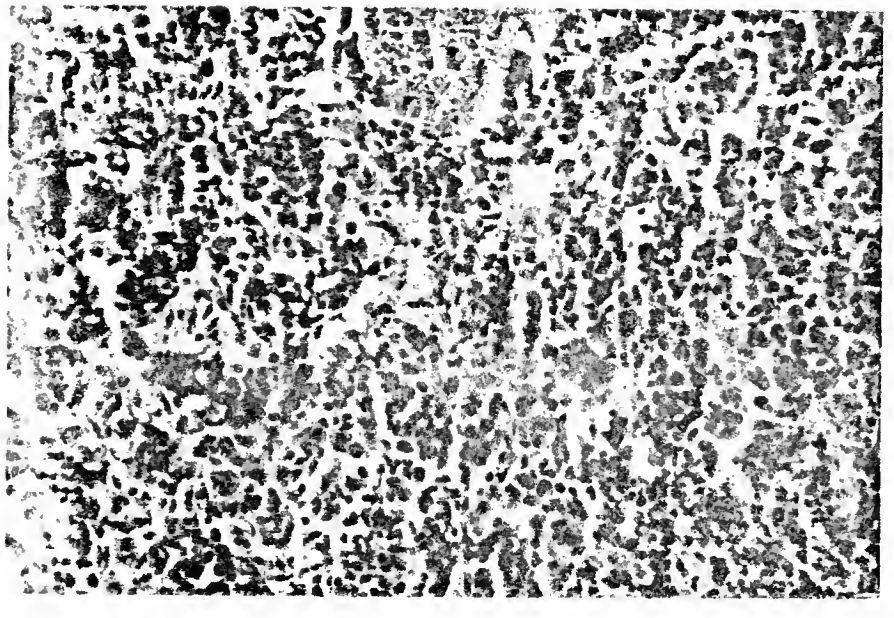
Separation and Quantitation of Bluegill Lymphocytes

Hypaque-Ficoll (ρ = 1.077) was used to siolate relatively pure populations of lymphocytes (characterized morphologically) from the

Figure 1. Photomicrographs of representative serial sections through the gill region of a small bluegill. (a) Anterior kidney. (b) Thymus. Sections were stained with hematoxylin and eosin. Magnification x 100.



b



a

blood, spleen, anterior kidney, and thymus of bluegill. Less than 5% of the total number of cells recovered from Hypaque-Ficoll were RBC's and the number of lymphocytes recovered represented at least 99% of the lymphocytes present in unfractionated whole blood or lymphoid organ cell suspensions.

White cell differentials of whole blood before and after fractionation on Hypaque-Ficoll are presented in Table 1 and illustrate the efficiency of this technique in removing other cell types. Figure 2 is a photomicrograph of the type of lymphocyte preparations routinely obtained from blood or lymphoid organ cell syspensions. These cell separations were successful, only if freshly caught fish were used. Another major cell type, a lymphoblast-like cell, was isolated from Hypaque-Ficoll if cell suspensions from fish maintained in laboratory aquaria for long periods of time were used (see Figure 3). The relevance of these blast-like cell isolates and the necessity of using newly acquired fish for these and subsequent studies is discussed in a later section.

The anterior kidney was the most abundant source of lymphocytes (yielding $\sim 2 \times 10^8$ cells/fish) whereas spleens and thymuses routinely yielded about 5×10^7 and 2×10^7 cells/fish, respectively. Heparinized blood yielded about 5×10^6 cells/ml (see Table 10).

Culture Conditions and Assay of Cell Division

As in any study involving *in vitro* culturing of lymphocytes (or any other cell type for that matter) there were numerous variables to be considered. In light of the fact that relatively limited numbers of cells were available from individual fish and since syngeneic bluegills were not obtainable it was necessary to approach optimization of culture

Table 1

White Cell Differentials of Bluegill Whole Blood and Hypaque-Ficoll Isolated Blood Cells

Cell Type[a]	Percent of Total[b]	
	Blood	Hypaque-Ficoll Isolated
Thrombocyte	25±5[c]	0
Granulocyte	30±4	0
Lymphocyte	45±5	100

(a) Smears were made of whole blood and Hypaque-Ficoll isolates of individual samples and were May-Grunwald-Giemsa stained for quantitation purposes.
(b) Results are expressed as a percent of the total number of white blood cells counted.
(c) Each value represents the mean of determinations from 6 different bluegill samples (>3 determinations per samples) ± standard deviations.

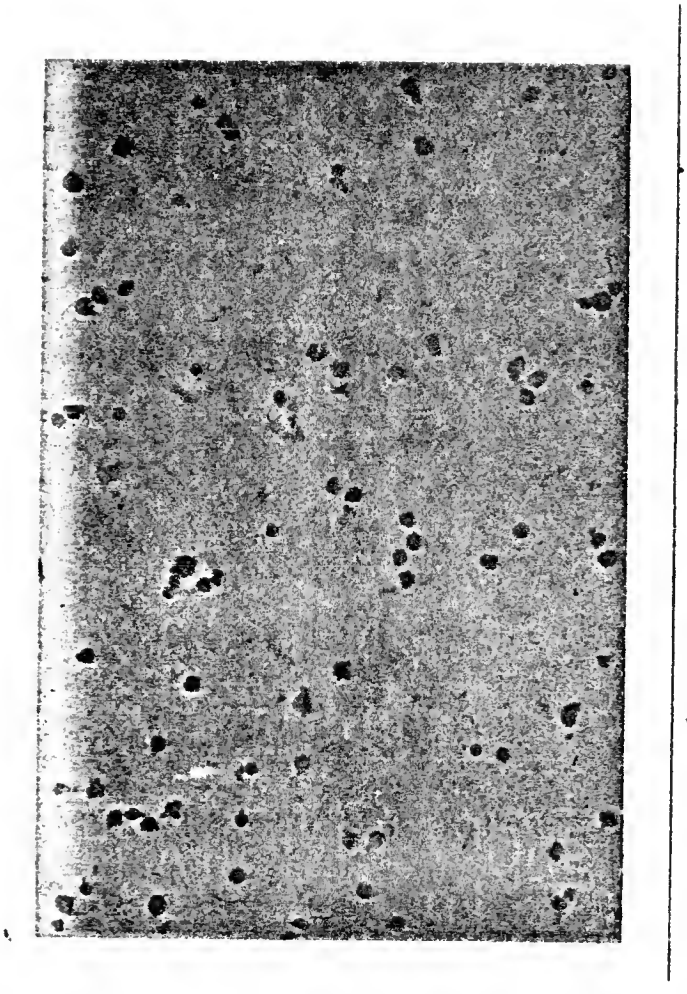


Figure 2. Photomicrograph of a representative Hypaque-Ficoll isolate from bluegill blood. A cytocentrifuge preparation stained with May-Grunwald-Giemsa. Magnification x 400.

conditions in a rather "piecemeal" fashion over an extended period of time. The following commentary is an effort to systhesize the major observations that enabled the definition of what can be called optimal conditions. Unless otherwise noted all of these studies were performed with anterior kidney lymphocytes.

Various sera or plasma were tested to determine which was a suitable supplement to use with RPMI 1640 for mitogenic studies of cultured bluegill cells. Ten percent human, calf, fetal calf, rabbit, alligator, bass, catfish, and grouper sera or plasma and mixtures of 5% human serum with 5% calf or fetal calf serum were not supportive in mitogenic stimulation studies using PHA (0.1 µl), Con A (10 µg) or LPS (1 or 10 µg) at either 22, 27 or 32°C. Grouper and catfish sera were cytotoxic for bluegill cells. The other sera gave high TCA precipitable counts in unstimulated control cultures and stimulation indices for mitogen stimulated cultures of 1 or < 1. On the other hand, bream (a collective term for all Lepomis species) serum pools were supportive in the sense that significant stimulation indicies were obtained with mitogen stimulated cultures.

In the initial experiments 10% bream serum was used. However, due to the limited supply of bream sera and the difficulty in obtaining good yields of serum from clotted blood, two modifications were tried and found satisfactory; 1) heparinized plasma rather than serum was used and 2) the concentration of supplement was reduced from 10% to 7%.

An additional complication was the observation that not all bream plasma pools were suitable as supplements in mitogenic assays. Variations in TCA precipitalbe counts of unstimulated control cultures ranged from < 100 CPM to > 10,000 CPM and stimulation indices varied from 4 to

250. One attempt to reduce the high counts of control unstimulated cultures obtained with some of the supplement pools was to dialyze the plasma pools against 0.15M NaCl. The data obtained with four bream plasma pools which elicited high background levels prior to dialysis arepresented in Table 2. In three of the four pools tested in this experiment the control CPM dropped significantly ($p < 0.05$) in the cultures incubated at 22°C and thus resulted in increased stimulation indices with LPS. With three of four pools used with cells maintained at 32°C the background remained unchanged. In the other case the background dropped as a result of dialysis and hence the stimulation index obtained using PHA increased. In conclusion it can be stated that dialysis of bream plasma did not significantly decrease the responses in any cultures and in fact in some cases enhanced the response. Thereafter all bream plasma were dialyzed before use as culture medium supplements for mitogenic assays.

Dialysis of certain heterologous supplements that elicited high control CPM was also tried. Dialyzed bass plasma was supportive as a supplement in mitogenic assays in the sense that significant stimulation indicies were obtained. However, these indices were never > 10 and therefore bass plasma was not used routinely. Dialysis of human, calf, fetal calf, and alligator sera did not improve the situation with respect to high levels of background counts.

To summarize the culture conditions discussed thus far, RPMI 1640 supplemented with 7% dialyzed bream plasma was found to be supportive for *in vitro* mitogenic stimulation.

During the course of several experiments involving different fish it was observed that there were differences both in the types of Hypaque-

Table 2

Effect of Dialysis of Plasma Supplements on Mitogenic Stimulation of Bluegill Anterior Kidney Lymphocytes

Supplement Plasma Pool[b]	Stimulation Index[a]			
	PHA (0.1 µl) - 32°C		LPS (1 µg) - 22°C	
	Undialyzed	Dialyzed[c]	Undialyzed	Dialyzed
A	1	6.8	1	1
B	4.8	4.0	1	14.0
C	2.5	4.0	5.9	16.4
D	4.8	4.6	1.5	7.4

(a) Triplicate cultures were stimulated with either PHA (0.1 µl) at 32°C or LPS (10 µg) at 22°C, pulsed on day 6 and harvested on day 7.
(b) Each pool represented the plasma obtained from at least five bream.
(c) Dialysis was against pyrogen free 0.15 M NaCl.

Ficoll isolated cells from anterior kidney cell suspensions and in the stimulation indices with mitogens. Furthermore these differences appeared to be correlated with the length of time the bluegills were maintained in holding tanks in the laboratory prior to sacrifice. Table 3 presents data on white cell differentials of Hypaque-Ficoll isolated cells from anterior kidneys of fish sacrificed at either one day or three weeks after capture. Significant increases in the number of blast-like cells were seen in cell preparations from bluegills maintained for three weeks. The gross differences in the cell types isolated from Hypaque-Ficoll can be seen by comparing the cells shown in Figure 2 (from a one day isolate) with those in Figure 3 (a three-week isolate). An increase in the number of red cells, which would not penetrate the Hypaque-Ficoll, was also noted in the three-week isolates.

Furthermore, it was also observed that in experiments utilizing fish maintained in acquaria for long periods of time, TCA precipitable counts of control unstimulated cultures were high. A composite of data from five experiments in which bluegill were sacrificed at various periods of time after capture is presented in Table 4. Apparently the longer a fish is maintained under our laboratory conditions the more likely it is the animal's lymphocytes will exhibit a high level of spontaneous thymidine incorporation. It thus seems imperative to use freshly caught fish as sources of cells for in vitro studies if the alternative is to keep them under the conditions used here.

To determine if TCA precipitable counts were a valid measure of cellular events in culture, TCA precipitable counts were correlated with the actual number of cells containing labeled thymidine. The technique of autoradiography was used. Anterior kidney lymphocytes

Table 3

Effect of Maintenance Time of Bluegill in Laboratory Aquaria on Differential White Cell Counts of Hypaque-Ficoll Isolated Anterior Kidney Cells

	Percent[a]	
Maintenance Time	Lymphocyte[b]	Blast-like
1 day	100	0
3 weeks	50-60	50-40

(a) Results are expressed as a percent of the total number of white cells counted.
(b) Cytocentrifuged preparations of Hypaque-Ficoll separated blood samples were stained with May-Grunwald-Giemsa for quantitation.

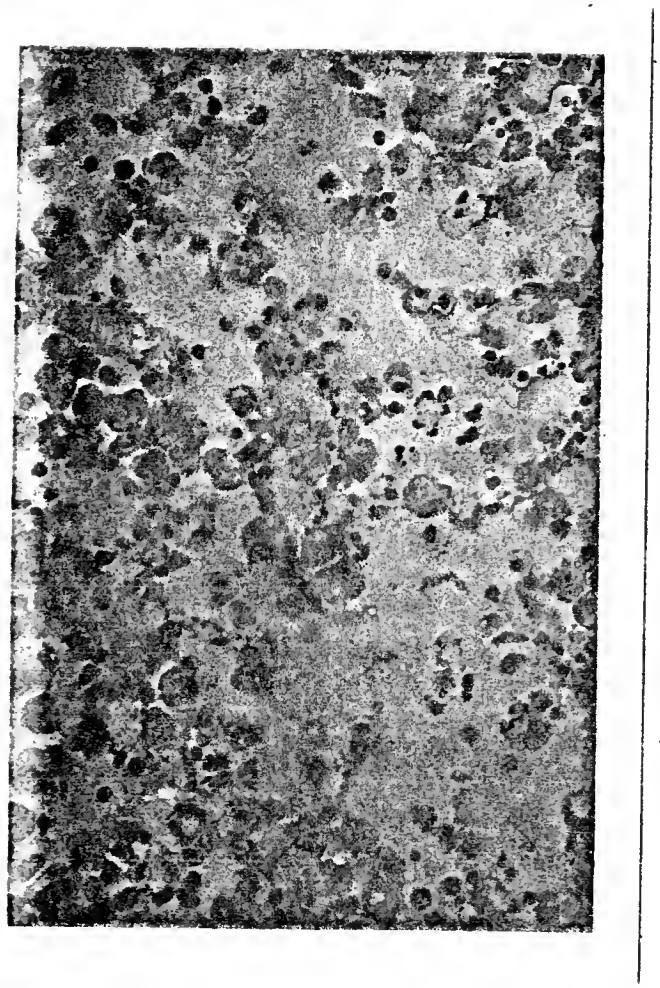


Figure 3. Photomicrograph of a representative Hypaque-Ficoll isolate of bluegill blood after long term laboratory maintenance of the bluegill. Photomicrograph is of a May-Grunwald-Giemsa stained cytocentrifuge preparation of an isolate obtained from a bluegill after three weeks of laboratory maintenance. Magnification x 400.

Table 4

Effect of Maintenance Time of Bluegill in Laboratory Aquaria on the Incorporation of Thymidine by Unstimulated Anterior Kidney Cell Cultures

Fish[b]	Maintenance Time[c]	CPM/Culture[a] 22°C	CPM/Culture[a] 32°C
A	1 day	50±8	45±8
B	1 day	120±14	44±6
C	3 weeks	1121±37	2645±109
D	3 weeks	991±68	1231±127
E	5 weeks	13,197±580	2838±181

(a) Results are expressed as the means of CPM from triplicate cultures ± standard deviations.
(b) Cells from individual fish were incubated without mitogens at 22°C or 32°C, pulsed with ^{3}H-thymidine on day 2 and harvested on day 3.
(c) Length of time fish were maintained in laboratory aquaria before sacrifice.

were cultured for seven days with various doses of PHA at 32°C. Tritiated-thymidine was added to all Cultures 24 hr prior to termination. One set of cultures was routinely harvested and assayed for TCA precipitable counts. Cytocentrifuge preparations were prepared from cells of a duplicate set of cultures and either processed for autoradiography and the number of labeled cells ($\geq$ 5 grains) quantitated (presented as a percent of the total number counted) or stained with May-Grunwald-Giemsa for morphological examination. As seen in Figure 4, increases or decreases in TCA precipitable radioactivity closely followed changes in the percent of the total number of cells that contained labeled thymidine.

In cultures stimulated with the optimal concentration of PHA (0.1 µl), 70% of the cells possessed nuclear autoradiographic grains. All labelled cells examined in toluidine-stained cytocentrifuged preparations were large blast-like cells and were found in clumps or aggregates (Figure 5a). Figure 5b is a May-Grunwald-Giemsa stained preparation showing an aggregate of blast-like cells with eccentric nuclei and abundant cytoplasm.

Mitogenic Studies

Experiments were designed to assess the optimal culture conditions for lymphocytes isolated from the anterior kidney. Variables tested were mitogen doses, time for maximum stimulation and effect of temperature on the response to the mitogens.

Figure 6 depicts the results of one very large study on the responsiveness of anterior kidney lymphocytes to LPS, PHA, and Con A under a variety of conditions. One somewhat surprising result involved the

Figure 4. Correlation of TCA precipitable counts with the number of autoradiography positive cells from PHA stimulated bluegill lymphocyte cultures. Cultures were incubated at 32°C, pulsed on day 6 and assayed on day 7.

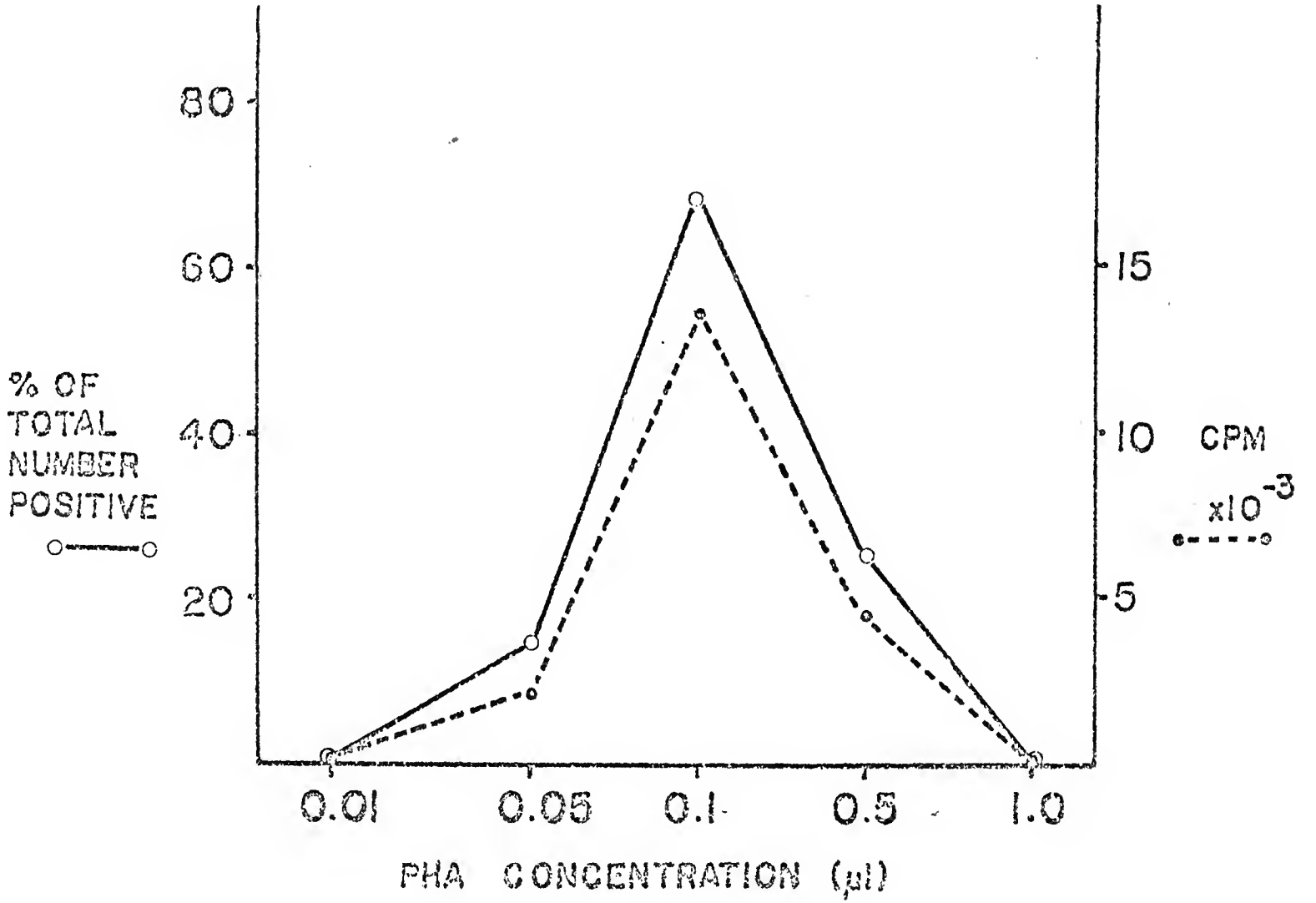
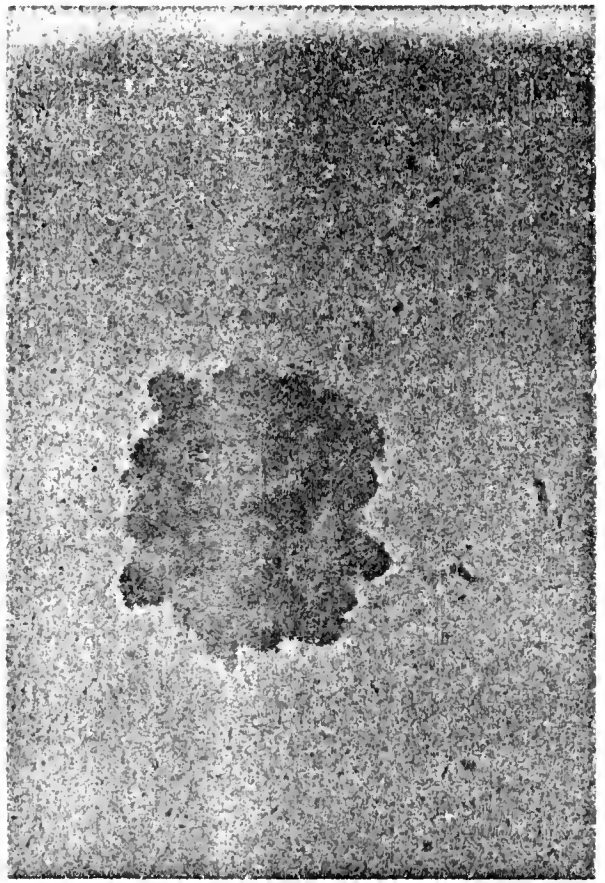

80
60
40
20
% OF TOTAL NUMBER POSITIVE
15
10
5
CPM x10^-3
0.01
0.05
0.1
0.5
1.0
PHA CONCENTRATION (µl)

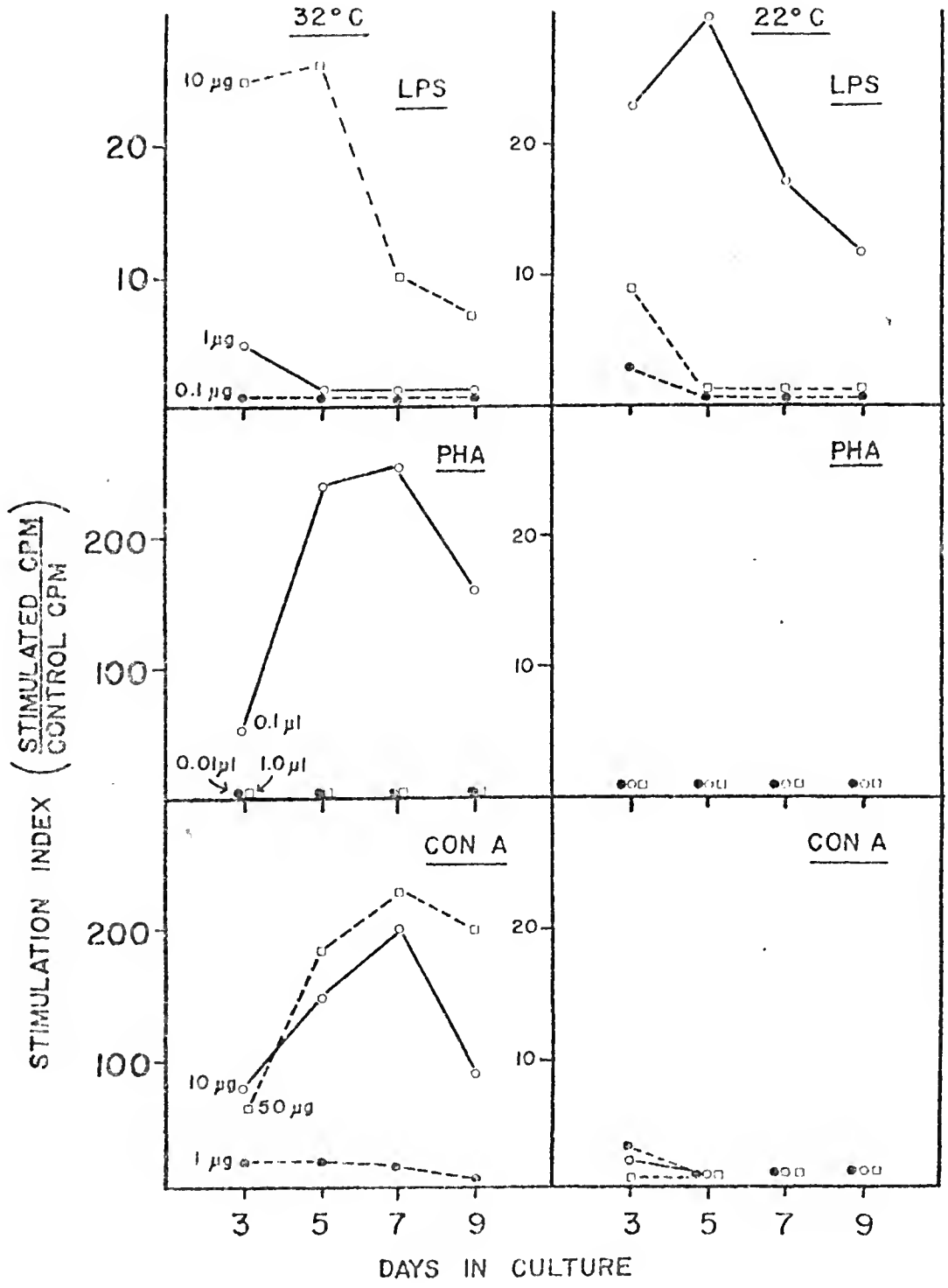
Figure 5. Photomicrograph of PHA-stimulated bluegill anterior kidney lymphocytes. (a) Autoradiograph stained with toluidine blue. (b) May-Grunwald-Giemsa stained. Magnification x 100.



a

b

Figure 6. Temperature effects on mitogenic responses of bluegill anterior kidney lymphocytes.


32° C
22° C
LPS
PHA
CON A
10 µg
1 µg
0.1 µg
0.01µl
0.1 µl
1.0 µl
50 µg
20
10
200
100
STIMULATION INDEX (STIMULATED CPM / CONTROL CPM)
3
5
7
9
DAYS IN CULTURE

differences in temperature on maximum stimulation with the various mitogens. Cells stimulated with PHA (0.1 µl) and Con A (50 µg) responded well at 32°C ($p < 0.01$) and very poorly, if at all, at 22°C ($p > 0.1$), whereas LPS (1 µg) responsiveness was higher at 22°C ($p < 0.01$). There was, however, a significant response ($p < 0.05$) to LPS (10 µg) in 32°C incubated cultures which was reproducible. Fifty micrograms of LPS (not shown) were not stimulatory (stimulation indices ≤ 1) at either temperature.

The temperature effects described above were found in ten experiments with the only major differences being the magnitude of the responses. These differences may have been due to differences in the serum supplement pools used as discussed previously.

To summarize the results, optimal mitogen doses at 32°C were 0.1 µl, 50 µg, and 10 µg for PHA, Con A, and LPS respectively and 1.0 µg of LPS at 22°C. PHA and Con A responses were greater at 32°C than 22°C and LPS responsiveness was greater at 22°C than 32°C. Optimal culture times were 5-7 days for all mitogens with the exception of 10 µg of LPS at 32°C where some variations were noted.

Limited experiments with spleen, blood, and thymus lymphocytes indicated that all were stimulated by PHA, Con A, and LPS. The mitogenic responses of thymus lymphocytes are presented in Table 5 to demonstrate that the temperature effects on mitogenic stimulations were also observed with cells from this tissue and thus were not limited to anterior kidney lymphocytes.

Mixed Lymphocyte Cultures

Lymphocytes from anterior kidneys of different bluegills were tested for their ability to respond in two-way mixed lymphocyte cultures

Table 5

Mitogenic Responses of Bluegill Thymus Lymphocytes

	Stimulation index[a]	
Mitogen	32°C	22°C
LPS (1 μg)	4.7	10
PHA (0.1 μl)	30	4
Con A (10 μg)	53	7

(a) Triplicate cultures were pulsed on day 6 and harvested on day 7.

(both populations capable of responding) at 22°C and 32°C. Cultures were initiated with 0.25 x 10^6 lymphocytes from each donor fish per culture well (0.5 x 10^6 cells total). Controls in mitogen stimulation studies for each fish also served as controls for mixed lymphocyte cultures.

Four of ten two-way mixed lymphocyte cultures exhibited statistically significant responses ($p < 0.05$) and are presented in Table 6. Significant responses were only obtained at 32°C thus mimicking responsiveness to PHA and Con A in temperature sensitivity. Furthermore, these studies have indicated that maximal stimulation (not shown) in the mixed lymphocyte cultures occurred at seven days. In this experiment all six bluegills studied had significant mitogenic responses, indicating there is no direct correlation between PHA and LPS responsiveness and the ability to respond to a mixed lymphocyte culture.

Evidence for Different Populations of Bluegill Lymphocytes

Golub (50) has demonstrated that rabbit anti-mouse brain cross reacts with mouse thymocytes due to a common antigen on both brain and thymocytes. In an attempt to elicit antiserum capable of recognizing antigenic surface determinants on bluegill lymphocytes a rabbit was hyperimmunized with bluegill brain homogenates following Golub's immunization procedures. To determine the specificity of this rabbit anti-brain serum for bluegill anterior kidney lymphocytes, cells were incubated with the rabbit serum and guinea pig serum. After appropriate incubation and washing only about 30% of the original number of cells remained viable (as determined by trypan blue exclusion) in contrast to 100%

Table 6

Mixed Lymphocyte Responses of Bluegill Anterior Kidney Lymphocytes

		Stimulation Index[a]					
		Mixed Lymphocyte Response		Mitogen Response			
				22°C		32°C	
Fish	Cross[c]	22°C	32°C	LPS[b]	PHA	LPS	PHA
1	1+2	1	25	62	2.3	9	95
2				17.2	7.2	8.9	145
3	3+4	1	12	22	16	3.4	51
4				20	3	2.4	55
3	3+5	1	9	22	16	3.4	51
5				24	2.9	5.3	22
4	4+5	1	19	20	3	2.4	55
5				24	2.9	5.3	22

(a) Triplicate cultures were pulsed on day 6 and harvested on day 7. Results are expressed as stimulation indicies as defined in Materials and Methods.
(b) Mitogen concentrations were 1 µg and 10 µg of LPS at 22°C and 32°C, respectively, and 0.1 µl of PHA at 22°C and 32°C.
(c) Designates the source of cells used in the mixed lymphocyte cultures.

recovery of viable cells when preimmune serum from this rabbit was employed as a control. When the cells surviving the rabbit antiserum treatment were assayed for mitogen responsiveness, it was found that the PHA response was diminished and the LPS response was intact. Results from two such experiments are presented in Table 7. These data indicate that cytotoxic treatment of anterior kidney lymphocytes with anti-brain plus complement may be an effective means of obtaining relatively pure LPS responsive cells and that this responsive population, representing ∿30% of the original population, may be a subpopulation of lymphocytes in the bluegill.

Anterior kidney lymphocytes were tested with heterologous red blood cells for spontaneous rosette formation. Results are presented in Table 8. Only rabbit red blood cells were capable of rosetting a significant portion of the lymphocytes (∿ 20%).

To determine if the rosetted lymphocytes represented a discreet subpopulation of the total with respect to mitogen responsiveness, rosetted cells were depleted from the non-rosetted ones using Hypaque-Ficoll centrifugation. Seventy to 75% of the original number of lymphocytes were recovered as non-rosette formers and were cultured under optimal mitogenic conditions. The results of two experiments are presented in Table 7. The LPS response was diminished while the PHA response was left intact.

These results indicate that depletion of lymphocytes rosetted with rabbit red blood cells from non-rosetted lymphocytes may be an effective means of isolating relatively pure PHA-responsive lymphocytes and that this responsive population, representing 70-75% of the original population, may be a subpopulation of lymphocytes in the bluegill.

Table 7

Mitogen Responses of Bluegill Anterior Kidney Lymphocytes Treated with Anti-Brain Plus Complement or Rosette Depleted with Rabbit Red Blood Cells

	Stimulation Index[a]					
	Expt 1		Expt 2		Expt 3	
Treatment	LPS[b]	PHA	LPS	PHA	LPS	PHA
Control	4.2	9.8	4	12.4	8	9.9
Anti-Brain + Complement[c]	30	1.3	26	1.3	ND	ND
Rosette Depletion[d]	1.8	17	ND[e]	ND	1	9.0

(a) Triplicate cultures incubated at 32°C, pulsed on day 6 and harvested on day 7.
(b) Mitogen concentrations were 10 µg and 0.1 µl of LPS and PHA respectively.
(c) A 1:5 of rabbit anti-bluegill brain plus a 1:10 of guinea pig complement was used in the cytotoxic treatments.
(d) Rabbit red blood cell rosetted cells were depleted by Hypaque-Ficoll centrifugation.
(e) ND = Not Done.

Table 8

Rosette Formation of Bluegill Anterior Kidney Lymphocytes with Red Blood Cells from Heterologous Species

RBC Source	% Rosetting[a]
Bluegill	0
Human	0.65 ± 0.2
Ferret	0
Alligator	0
Rabbit	21 ± 1.5
Guinea Pig	0.75 ± 0.2
Horse	0
Mouse	0
Sheep	1.3 ± 0.6
Chicken	2.5 ± 1.0

(a) Results are expressed as percentages of the total number of bluegill white cells (total number of white cells minus the number of white cells in the RBC controls) rosetting with the red blood cells. Each value represents the mean of triplicate determinations from three different fish ± standard deviations. A white cell in contact with $\geq$ 4 RBC constituted a positive rosette.

In Vivo and In Vitro Studies on Antibody Producing Cells

Prior to *in vitro* primary immunization studies with cell suspensions from the bluegill, it was first necessary to determine which organs contained antibody-producing cells. It was also necessary to determine if bluegill were responsive *in vivo* to the test antigen (SRBC) and to establish a suitable complement source for use in the hemolytic plaque assay.

Bluegill were immunized intraperitoneally with sheep red blood cells and sacrificed two weeks later. Cell suspensions were prepared from the anterior kidney, spleen, and thymus. Only blood was fractionated on Hypaque-Ficoll due to difficulties in assaying samples with a high ratio of red to white cells. Each cell suspension was assayed in a Jerne hemolytic plaque assay for cells producing antibody to sheep red blood cells.

Wide variations in responsiveness to sheep red blood cells were observed in immunized bluegill. Results from two individuals are presented in Table 9. The spleen, anterior kidney, and thymus each contained considerable numbers of antibody-producing cells. Each organ had approximately the same number of plaque-forming cells (PFC) per 10^6 cells. Very few plaque-forming cells were present in blood, though it should be emphasized that blood was fractionated on Hypaque-Ficoll prior to assay.

Fresh guinea pig serum, grouper serum, alligator serum, bass plasma, bream plasma, and sucker plasma were diluted 1:20 and used as sources of complement in the Jerne assay. Only bream, bass, and sucker sera were effective sources of complement. Since sucker plasma was not used as medium supplement and was obtainable in large quantities, it was used routinely as a complement source.

Table 9

Distribution of Antibody Forming Cells in Various Tissues of Bluegill Immunized with Sheep Erythrocytes

Fish[a]	Tissue	White[b] Cells ($x10^{-6}$)	Number of PFC	
			Per 10^6 Cells	Total
1	Blood (2 ml)	9	4	36
	Kidney	100	70	7000
	Thymus	18	53	954
	Spleen	9	52	468
2	Blood (2 ml)	10	1	10
	Kidney	184	3	552
	Thymus	30	4	120
	Spleen	24	2	48

(a) Bluegill were immunized intraperitoneally with 0.1 ml of 10% SRBC and were sacrificed after two weeks.
(b) Hypaque-Ficoll fractionated peripheral blood cells and unfractionated organ cell suspensions were assayed for the number of white cells and the number of plaque forming cells (PFC).

The number of cells in the various lymphoid organs containing cytoplasmic immunoglobulin were assayed by indirect immunofluorescence. Smears of washed, unfractionated cell suspensions of anterior kidney, thymus, spleen, blood, and posterior kidney (as a negative control) were examined and the number of cells showing positive cytoplasmic immunoglobulin staining quantitated. The results are presented in Table 10. The posterior kidney was devoid of any Ig-containing cells. Anterior kidney, spleen, thymus, and blood demonstrated appreciable numbers of immunoglobulin containing cells.

Preliminary studies were undertaken to determine if bluegill lymphoid cell suspensions would respond in vitro to an antigenic stimulus. Several modifications of the culture techniques discussed above for mitogen studies were employed to enrich the culture media and to ensure that all necessary cellular components were present.

Undialyzed 7% bass plasma rather than bream plasma was used as a supplement with an enriched RPMI 1640 medium. Since the hemolytic plaque assay only measured differences in the number of plaque-forming cells between control and antigen stimulated cultures, a high nonspecific stimulus by bass plasma (see mitogenic studies) was irrelevant as long as an increase in plaque-forming cells was attributable to antigenic stimulation.

A pool of unfractionated cell suspensions of anterior kidney, spleen, and thymus was used for three reasons: 1) to increase the number of available cells and thus the number of variables that could be tested, 2) to include phagocytic and plasma cells as well as any other cell types possibly involved in antigen processing and antibody formation, and 3) to decrease the chance of compartmental effects of individual

Table 10

Immunoglobulin Producing Cells
in the Lymphoid Organs of the Bluegill

Organ[a]	% Positive[b]
Blood	20 ± 5
Spleen	45 ± 11
Thymus	39 ± 15
Anterior Kidney	40 ± 14
Posterior Kidney	0

(a) Smears of blood and organ cell suspensions were assayed by indirect immunofluorescence for cytoplasmic immunoglobulin.
(b) Results are presented as a percent of the total number of white cells counted and are means of multiple determinations from three bluegill ± standard deviations.

organs. All cell suspensions used contained < 30% red blood cells and ∿ 7-10% phagocytic cells (determined by colloidal carbon untake).

Control (no SRBC) or immunized (with SRBC) cultures were assayed for PFC in the Jerne hemolytic plaque assay after incubation at 22°C and 32°C for various time periods. Two experiments utilizing unimmunized "normal" bluegill as cell donors are presented in Table 11. In 32°C incubated cultures there were significant increases in the number of PFC of immunized cultures over control culture responses. The maximum PFC response as well as the maximum number of recovered cells from immunized than control cultures occured on day 7. More cells were recovered from immunized than control cultures and on day 7 more than the initial (Day 0) number of cells were present in immunized cultures. Viability in the cultures did not change over the ten-day culture period.

In contrast to the 32°C incubated cultures, cultures maintained at 22°C did not show a PFC response. There was no significant difference between control and immunized cultures and the viability was lower after ten days.

One preliminary experiment was done with cells from an immunized bluegill in order to determine if a secondary immunization <u>in vitro</u> would increase the number of responsive cells. Unlike cells from normal fish, the PFC response in this fish was observed to occur only at 22°C. The magnitude of the response measured on day 7 however was much lower (control = 0 PFC, "boosted" = 18 PFC/Culture) than that seen at 32°C with cells from normal animals. It should be pointed out that the number of recovered cells in the single experiment conducted was higher in 22°C incubated cultures (22°C, 90% for controls, 285% for boosted;

Table 11

Primary In Vitro Immunization of Bluegill Lymphoid Organ Cell Suspensions with Sheep Red Blood Cells

Experiment	Culture	Days in Culture	PFC/Culture		% Recovered[b]		% Viable[c]	
			22°C[a]	32°C	22°C	32°C	22°C	32°C
1	Control	5	ND[d]	57	ND	95	ND	89
	Immunized	5	ND	660	ND	115	ND	93
	Control	7	0	50	72	49	88	96
	Immunized	7	0	1045	65	177	94	97
	Control	10	ND	38	ND	62	ND	86
	Immunized	10	ND	810	ND	101	ND	90
2	Control	7	0	0	83	56	89	91
	Immunized	7	0	147	77	109	95	93
	Control	10	0	0	36	40	73	94
	Immunized	10	0	82	36	68	69	90

(a) Cultures were maintained at the indicated temperatures.
(b) Cell recoveries are expressed as a percent of the initial number of cells (Day 0).
(c) Viability was determined by trypan blue exclusion and is expressed as a percent of the total number of cells recovered from cultures.
(d) ND = Not Done.

32°C, 4% for controls, 60% for immunized). It thus seems possible that a major difference between the in vitro primary and secondary responses to sheep erythrocytes may exist although obviously more work needs to be done before definitive statements are possible.

Discussion

Effects of Plasma Supplements and Fish Maintenance on Lymphocyte Cultures

Two crucial variables, the medium supplement and the health or physiological state of the fish appeared to be critical in obtaining high levels of DNA synthesis (i.e. TCA precipitable counts) in unstimulated lymphocyte cultures. The causative factors in these two situations are unknown but it would seem appropriate to discuss, in a speculative way, these two points. The influence of serum factors on *in vitro* cultured cells has been well documented in other systems (18,38, 64,85,99,100,101,119,120) and it is conceivable in the studies reported here that one or more such factors were present in some of the plasma pools used as supplements. Dialysis experiments suggest that a factor(s) of < 10,000 molecular weight was responsible for nonspecifically elevating unstimulated control TCA precipitable counts. It is also interesting that Etlinger's mitogenic studies with rainbow trout leukocytes (46) also revealed serum effects on stimulation indicies.

Numerous effects on fish subjected to environmental changes or stress have been reported (23,104). For example, physiologically significant serum alterations in cortisol, glucose, and free fatty acid levels as well as morphological changes in adrenocortical, medullary, and pancreatic tissues occur within minutes in goldfish subjected to the slight stress of an aquarium transfer. The effects of environmental factors, other than temperature, on the immune responses of fish have,

however, not been studied. The data presented here suggest that the altered cellular-state (an increase in blast-like cells concommitant with an increase in TCA precipitable counts of unstimulated cultures) in bluegill maintained for long laboratory periods are caused by environmental factors in the laboratory aquaria. A likely factor (admittedly speculative) might involve endotoxemia resulting from bacterial infections acquired in the aquarium.

Evidence for Two Subpopulations of Lymphocytes

The studies reported here show that there are at least two subpopulations of lymphocytes in the bluegill. One population is stimulated by PHA and Con A at 32°C and very poorly at 22°C. Although not proven directly, the cells responding in mixed lymphocyte cultures are probably a subset of the PHA/Con A responsive population since MLC's were obtained only at 32°C. The other population of lymphocytes is LPS-responsive at both 32°C and 22°C although responsiveness at 22°C was usually greater.

The two subpopulatins were shown to be different by anti-brain serum cytotoxicity and rosette depletion experiments. The 32°C, PHA responsive population was depleted from the total population by anti-brain plus complement treatment and left intact by depletion of rabbit RBC rosetted lymphocytes. The converse was true for the LPS-responsive population. LPS-responsiveness was depleted by removal of rosetted lymphocytes from the total population and was unaffected by anti-brain cytotoxicity treatments.

Comparison of Bluegill and Rainbow Trout Mitogenic Studies

Differences between the results of mitogenic studies presented here with the bluegill and those of Etlinger et al. (46) with rainbow

trout leukocytes suggest that there may be major differences between different species of fish. Unlike the bluegill, rainbow trout contained PHA-responsive cells only in the thymus and LPS-responsive cells only in the anterior kidney in a manner analogous to the compartmental localization of T- and B-cells in birds and mammals. However, accurate comparisons of the rainbow trout and bluegill are tenuous due to experimental differences. Unfractionated leukocytes, rather than isolated lymphocytes, were cultured only at 19°C in the trout studies. It was also deemed necessary to switch serum supplements to obtain significant responses to different mitogens with trout cells. There were also differences in optimal mitogen doses as well as length of time for maximum mitogenic stimulation between the two species.

It is thus conceivable that true differences in the lymphoid systems exist between different species of fish. For example, there are reports that thymuses of some fish species involute with age while others do not (37). It is suggested that a third species group may exist in which the thymus differentiates (or de-differentiates) into a lymphoid organ similar to the anterior kidney, as apparently is the case with bluegill.

Differences in environmental temperature tolerances may also effect the in vitro cellular responses. Rainbow trout live in colder environments, and thus evolutionary pressures may have affected the subpopulations of lymphocytes to a point where discernible differences in in vitro temperature responses may not be recognizable. Further in vitro studies with other species are necessary before adequate comparisons of this nature can be made.

Are Bluegill Lymphocyte Subpopulations T- and B-Cell Equivalents?

By analogy, the mitogenic and mixed lymphocyte culture responses of bluegill lymphocytes would support the conclusion that fish have T- and B-cells. Bird and mammalian T-cells respond to PHA and Con A (but not LPS) and are reactive in mixed lymphocyte cultures. Similarly, a bluegill lymphocyte subpopulation (depleted of rabbit RBC rosettes) responds to PHA or Con A when cultured at 32°C. The MLC reactive cells also responded only at 32°C and are probably a subset of the PHA/Con A reactive cell population. Bluegill lymphocytes of the subpopulation unaffected by anti-brain plus complement treatment responded only to LPS, and B-cell mitogen in birds and mammals. However, such conclusions should be approached with caution until functional activities are associated with the two bluegill lymphocyte subpopulations.

It should also be pointed out that the spontaneous rosette formation of the B-like cells with rabbit RBC's is in marked contrast to all other animal species studied, in which the B-cells do not spontaneously rosette with any RBC's.

Implications of In Vitro Studies

If one assumes that *in vitro* studies are valid measures of *in vivo* events, several explanations or rationalizations of published *in vivo* data are possible in light of the *in vitro* temperature effects on bluegill lymphocytes.

Numerous reports on the effects of temperature on the immune responses in fish to bacterial or protein antigens have been published. Avtalion *et al.* (7) have suggested that the effects can be explained by two populations of lymphoid cells; one is the antigen-reactive population requiring a higher temperature to process the antigen and the other

population is responsible for antibody production at either high or low temperatures. This may be the case if indeed the PHA-(and Con A-) responsive cell is equivalent to the antigen reactive cell and the LPS-responsive cell is equivalent to the antibody-producing cell.

The participation of the two defined cell populations and the temperature effects on immune responses of bluegill should be testable in vitro. In vitro SRBC primed cultures maintained at 32°C elicited a very good plaque forming cell response to SRBC's whereas cells maintained at 22°C gave no response. If the SRBC is a T-dependent antigen in the bluegill, as in mammalian systems, then application of depletion techniques (rosette depletion or anti-brain cytotoxicity) should demonstrate whether cellular cooperation between the two subpopulations is involved in in vitro antibody production. Further application of in vitro manipulation techniques to the hapten-carrier effect should also establish if the two subpopulations are indeed T-like and B-like in function.

The preliminary study utilizing cells from in vivo primed fish also were supportive of Avtalion's conclusions that fish can respond to a secondary antigenic challenge at low temperatures only if they are primed at a higher temperature. In vitro "boosted" cells responded at 22°C, though with lower numbers of plaque-forming cells. However 32°C cell cultures were not responsive, contrary to in vivo primary immunization studies. This may indicate a secondary antigenic stimulus at 32°C which elicits a tolerant state or suppressive factor(s).

Yocum et al. (121) have shown that only 16S IgM-like antibody is produced in the hapten-carrier effect in a marine fish, the searobin. Apparently the switch from high molecular weight to low molecular weight

antibodies (a T-cell controlled event in mice) associated with the hapten-carrier effect in mammals does not necessarily occur in fish. However, Uhr et al. (113) demonstrated that goldfish, when acclimated to a high temperature (35°C), were capable of responding to an antigen with both 16S and 7S antibodies (as opposed to a response at lower temperatures of only 16S antibodies). Though it was not proven that the 7S antibody was in fact a de novo product and not a degradation product of the 16S antibody or a shed membrane receptor, a PHA, high temperature responsive cell type conceivably could be functional in controlling the switch mechanism at 35°C in goldfish.

Temperature effects on lymphocytes may not be confined solely to bluegill lymphocytes. R. C. Ashman, University of Western Australia, Nedlands (personal communication) has demonstrated an increase in PHA responsiveness of human T-cells when cultured at 39°C rather than 37°C. Armadillos have body temperatures of < 35°C, yet the transformation of lymphocytes stimulated by PHA was increased approximately 2.6 times when cultured at 37°C rather than 33°C (91). Perhaps an evaluation of mitogenic responses of other mammalian lymphocytes cultured in narrower temperature ranges (37° ± 2°C) is warranted. However experiments done by J. W. Shands, Jr., University of Florida, Gainesville, Fla. (personal communication) using mouse spleen lymphocytes cultured with LPS and PHA at 22, 27, 32, 35, 37 and 39°C showed the optimal response to both mitogens was obtained at 37°C.

The Bluegill Lymphocyte as an Experimental Model

Differential responses to mitogens by cells cultured at different temperatures should provide a valuable method to study functional and physiochemical properties of the cells involved in immune reactions of

fish. One could speculate that the temperature effects on lymphocytes cultured with mitogens are due to changes in membrane fluidity. Theoretically a more rigid membrane in a PHA-responsive, 22°C cultured lymphocyte could inhibit capping and membrane events leading to cell activation, whereas a PHA-responsive, 32°C cultured cell with a more fluid membrane could respond. Changes in membrane fluidity would also account for changes in optimal doses of LPS required at the different temperatures. Experiments to chemically alter membrane rigidity would test the concept of temperature sensitive events at the membrane level.

There are alternative explanations for the temperature effects demonstrated with bluegill lymphocytes, such as conformational changes in receptor molecules with changes in temperature or the influence of temperature on intracellular events involved in cell activation. In any event, the question of why the two subpopulations differ in responsiveness at different temperatures is an intriguing one. It would appear that fish may offer a unique approach to dissecting cellular events in the immune response.

CHAPTER III
LYMPHOCYTE HETEROGENEITY IN THE ALLIGATOR

Introduction

The reptiles are thought to represent a pivotal point in the phylogeny of the immune system since phylogenetically they are a common ancestor of the birds and mammals. However, as pointed out by Cohen (36), immunological studies in the reptiles are severely lacking. The available data, reviewed in (36,37,59), suggest that reptiles can mount a diversity of immune responses and arguments by analogy would suggest they likely have T-like and B-like cells lymphocytes.

Various antigens have been used to elicit both primary and secondary humoral responses in various reptilian species (36,37,56,72) with a switch from 19S IgM-like antibody molecules to 7S IgG-like antibody molecules occurring during secondary responses (4,56). Unfortunately relatively little has been done to describe the heavy chain isotypes in the reptiles and thus IgM and IgG (or IgY) designations are at best tenuous (31). Cells resembling plasma cells have been detected by fluorescent antibody techniques, electron microscopy and the Jerne plaque assay (36) in turtles. Thus on the basis of the ability to elicit antibody responses as well as the demonstration of plasma-like cells involved in antibody production, the evidence is rather direct that reptiles have a B-cell equivalent.

First and second-set skin allograft rejections (37,59) characteristic of T-cell reactions in mammals have also been demonstrated in reptiles with an anamnestic second-set response. However there is a major difference between transplantation reactions of reptiles and mammals, in that reptilian reactions are typically chronic (36,37) as opposed to the acute rejections occuring in mammals. These data suggest that T-like functions may differ from those in mammals. Indeed, graft rejection sites in turtles and snakes are infiltrated very early not only with lymphocytes and macrophages, but also with an abundance of plasma cells (11). This observation suggests that such chronic graft rejections may be antibody-mediated rather than cellularly (via T-like cell) mediated. Responses to haptens conjugated to protein carriers have also been demonstrated in reptiles (8,73) although the hapten-carrier effect has apparently not been studied. In brief, data demonstrating that reptiles can 1) show a 19S to 7S switch, 2) produce anti-hapten antibodies, and 3) undergo graft rejections are at best only circumstantial evidence for the existence of a T-like cell in these species. In fact one could conceivably (although perhaps not too convincingly) argue for the existence of only B-like cells from the same data.

Many of the reports from previous in vivo experiments in which humoral responses to antigenic challenge were tested conflicted with one another and in some cases there were questions as to whether reptiles could respond to antigenic challenges at all (36). Many of these discrepancies have since been attributed to differences in the temperatures at which the animals were maintained after immunization. As early as 1901, Metchnikoff demonstrated that the alligator responded to diphtheria toxin by forming antitoxin if the alligators were maintained at

32-37°C, whereas at 22°C they did not respond at all (80). More recently, Evans has presented evidence that desert lizards maintained at 35°C responded well to sheep red blood cells, but if maintained at 30°C or 40°C, temperatures well within physiological temperature ranges, they did not respond as well (47). Also, an active humoral response to the antigen was stopped if the animals were moved from 35°C to the lower temperature. Wetherall and Turner (118) observed similar responses to changes in environmental temperatures in another lizard species. Environmental temperature is also an important factor in skin allograft rejections, as shown by Borysenko (11). Snapping turtles accepted allografts when they were maintained at 10°C but were able to reject the allografts at 25°C, and more rapid rejections were seen at 35°C.

The lymphoid organs of several representative reptilian species have been examined histologically (36,37). A bursa, thymus, spleen, and gut associated lymphoid aggregates have been demonstrated. However the functional roles of the various organs are lacking and thus it cannot be stated whether the "bursa-like" organs are sources of B-like cells or that the thymocytes are T-like cells as seen in the chicken. In immunized turtles antibody-forming cells were found in the spleen but not in the thymus (36), but again, the data are only circumstantial that the lymphoid organs are compartmentalized into T- and B-cell components. To summarize the current literature, it would appear that direct evidence for two cell types in any reptile analogous to T- and B-lymphocytes in birds and mammals is lacking.

The purpose of this portion of the research was to determine in a direct way if a reptile, the Florida alligator, has a heterogeneous

population of lymphocytes akin to T- and B-cells. The approach taken was similar to that described previously for the bluegill, i.e. 1) to define a separation technique for the isolation of relatively pure lymphocytes and to establish appropriate *in vitro* culture conditions for these cells, 2) to determine if mitogen stimulation and cell surface antigens employed as T- and B-cell probes and membrane markers in the bird and mammalian systems are applicable to alligator lymphocytes as *in vitro* markers, and 3) to separate differing subpopulations of lymphocytes on the basis of marker differences. Special emphasis was also directed towards studying the effects of temperature on alligator lymphocytes to determine if a cellular basis for the *in vivo* temperature effects on the immune responses in reptiles could be demonstrated.

Materials and Methods

Experimental Animals

Florida alligators (Alligator mississippensis) were obtained from the Florida Game and Fresh Water Fish Commission. Male and female alligators, 90-150 cm in length, were used. Accurate age determinations were not possible, but were estimated to be between three and five years. Alligators were individually tagged and housed in a 1.5 m x 6 m outdoor pen at the University of Florida Animal Quarters. The pen was designed to provide the alligators with easy access to either water or a dry platform. The alligators were fed daily with monkey biscuits (Ralston Purina, St. Louis, Mo.) and to satiation twice each week with fresh fish (bream).

Culture Media

Culture media for in vitro mitogenic and primary immunization studies were as described in Chapter II with the following modifications: 1) Minimum Essential Medium (MEM) with nonessential amino acids (GIBCO) was substituted for RPMI 1640 and 2) the NaCl concentration of the complete media was increased to 0.157 M by dissolving 2.400 g NaCl in the medium prior to adjusting the final volume to 1.0 L. The prepared MEM containing extra NaCl was designated Gator MEM (G-MEM) to distinguish it from mammalian MEM.

The above modifications were also used in preparing medium used for in vitro primary immunization studies following the procedure presented in Chapter II.

Supplement Sources

Alligator, human, calf, fetal calf, and rabbit sera were tested as media supplements for *in vitro* studies. Two alligator serum sources were used: 1) eight different pools (> 10 individual bleedings, 30-40 ml of serum per animal) were obtained from 1-2 kg alligators (2-3 yr of age) at Herman Brooks' Alligator Farm (Christmas, Fla.) and 2) sera (100-250 ml serum per bleed) from individual 100-225 kg alligators (> 10 yr old) which were maintained at Silver Springs Reptile Institute (Silver Springs, Fla.). The remaining serum sources are indicated in Chapter II.

Preparation of Cell Suspensions and Counting Techniques

Lymphoid organs and cell descriptions are described in several references (25,36,77). Methods for the preparation of organ cell suspensions described in Chapter II were followed. Blood was drawn from the internal jugular vein into a heparinized syringe (50 U heparin/10 ml blood). This method of obtaining alligator blood was originally described by Herman Brooks (alligator farmer, Christmas, Fla.) and published by Olson *et al.* (86). A maximum of 5 ml of an organ cell suspension or undiluted heparinized whole blood was layered onto Hypaque-Ficoll (ρ = 1.077). Techniques for centrifugation, cell washes, cell counts, and viability determinations are described in Chapter II.

Culture Techniques

Culture techniques are described in Chapter II with the following additions or changes: 1) 10% alligator serum was used as a supplement, 2) two additional mitogens, pokeweed mitogen (DIFCO) and purified

protein derivative (a gift from Dr. R. Waldman, University of Florida) were used, and 3) only peripheral blood lymphocytes were used in in vitro primary immunization studies.

Assay for ^{3}H-Thymidine Incorporation into DNA

Assay techniques are described in Chapter II.

Stimulation Indices and Statistical Analysis

Statistical analysis and formulas for calculating stimulation indices are presented in Chapter II.

Autoradiography

Techniques for autoradiography are presented in Chapter II.

Histological and Morphological Techniques

Serial cross sections of paraffin embedded organs were kindly prepared by Mr. Larry J. McCumber (Whitney Marine Laboratory, Marineland, Fla.). Sectioned tissues, as well as cytocentrifuge preparations of cell suspensions, were stained with May-Grunwald-Giemsa stain.

Preparation of Rabbit Antisera

The brain of one sacrificed alligator was used for immunization purposes, following techniques described in Chapter II. Antisera from two rabbits immunized and boosted eight times over a four month period were used. Preimmune sera from the same rabbits were used as normal rabbit serum controls.

Rabbit anti-alligator immunoglobulin was prepared by immunizing rabbits with immunoglobulins isolated by Sephadex G-200 (Pharmacia) column chromatography. An ammonium sulfate precipitate of alligator serum was applied to the column.

Cytotoxicity Assay

The protocol described in Chapter II was followed.

Rosetting Techniques

The method of Jondal et al. (67) as described in Chapter II was used to assess the number of peripheral blood lymphocytes capable of rosetting with sheep red blood cells.

Immunofluoresence

The methods described in Nairn (84) were followed for indirect immunofluoresent stains of cytocentrifuge preparations of cell suspensions normal rabbit serum or rabbit anti-alligator immunoglobulin and a fluorescein labeled goat anti-rabbit IgG. Immunofluorescent methods for membrane stains are described in Chapter IV.

Hemolytic Plaque Assay

The techniques for harvesting cultured cells and assaying for plaque-forming cells are described in Chapter II. Fresh alligator serum diluted 1:20 was used as a complement source.

Cellular Immunoadsorbents

The method of Chess et al. (24) was used for fractionating alligator peripheral blood lymphocytes on cellular immunoadsorbents. Rabbit anti-alligator immunoglobulin was precipitated with 40% ammonium sulfate, washed three times and redissolved in 0.15 M NaCl. The immunoglobulin enriched fraction was then dialyzed against 0.15 M NaCl - 0.005 M $Na_2B_4O_7$ (pH 8.3) prior to coupling onto CnBr activated Sephadex G-200 (Pharmacia). Preimmune rabbit serum, treated in an identical manner, was coupled to Sephadex as a control.

Affinity columns were prepared as follows: The coupled Sephadex G-200 preparations were washed with 5% FCS in G-MEM and 8 ml of packed volume was poured under 1 x g into 12 ml disposable syringes. Two and one half billion cells in 2.5 ml of 5% FCS in G-MEM were loaded dropwise (10 drops/min) followed by the slow dropwise addition of 5% FCS in G-MEM. Elutions were monitored periodically until the effluent was cell free. The nonadherent cells were washed three times with medium prior to further use.

Glass Wool Fractionation

The method described by Trizio and Cudkowicz (110) was adapted for use in glass wool and nylon wool column fractionations of alligator peripheral blood lymphocytes. Glass wool (Corning Glass Works, Corning, N.Y.) was pretreated by rinsing three times with pyrogen free 0.15 M NaCl, boiled 1 hr in tripled-distilled water (three changes) and dried by lyophilization. Twelve milliliter disposable syringes were packed to the 8 ml mark with the pretreated glass wool and sterilized. Prior to loading cells on the prepared column, 40 ml of prewarmed (32°C) G-MEM was passed through the column followed by 15 ml of 5% FCS in G-MEM. The column was then incubated for 30 min at 32°C in 5% CO_2-95% air. One hundred million cells in 2 ml of 5% FCS in G-MEM were loaded onto each column and were washed into the column with 1 ml of 5% FCS in G-MEM. Loaded columns were incubated in a vertical position at 32°C for 1 hr in 5% CO_2-95% air. Nonadherent cells were eluted very slowly (20 drops/min) with 20 ml of 5% FCS in G-MEM (32 C). Fifteen milliliters of warm 5% FCS in G-MEM were then slowly flowed through as a "buffer" between the nonadherent and the adherent fractions. Care was taken not to generate a fluid head of pressure nor to jar the column during the slow

elution of the nonadherent cells or the "buffer" flow through. Adherent cells were eluted in a 40 ml volume of G-MEM by generating a fluid head of pressure as well as mechanically disrupting the glass wool. Cell fractions were washed three times prior to further analysis.

A procedure identical to that described in the preceding paragraph was followed in the preparation and use of nylon wool columns.

Results

Lymphoid Organs of the Alligator

Since the Florida alligator is listed by the Florida Game and Fresh Water Fish Commission as an endangered species, only a limited number of alligators were available for experimental purposes. Fortunately, it was easy to obtain large amounts of blood which was an abundant source of lymphocytes (1-2 x 10^7 lymphocytes/ml of whole blood). There were no detrimental effects to the animals. Evidence will be presented in a subsequent section that the population of lymphocytes isolated from peripheral blood are representative (on the basis of mitogenic responsiveness) of the lymphocytic cells isolated from the spleen.

Two of ten alligators obtained from the Florida Game and Fresh Water Fish Commission were sacrificed (by special permit) for histological examinations and *in vitro* mitogenic studies of the lymphoid organs. The only recognizable lymphoid organs were the thymus and the spleen. The thymus was a small whitish organ, approximately 2 x 8 mm located in the throat. Histological examinations of tissue sections showed an abundance of lymphocytes and signs of thymic involution were seen. Very few cells were isolated by Hypaque-Ficoll centrifugation from whole organ cell suspensions (< 5 x 10^6). The spleen of the alligator was a red, kidney-bean shaped organ, located beneath the stomach, and was surrounded by a thick capsule. Red and white pulp regions were observed in tissue sections and a heterogeneous population of white cells was

seen. Only 2-5 x 10^7 cells were isolated from Hypaque-Ficoll isolated preparations of whole spleen cell suspensions.

Small aggregates of lymphoid cells were present in glandular tissues found in the orbital sinus and the area of the cloaca. However further histological studies are necessary before these tissue can be defined as lymphoid equivalents of the Harder's Gland or Bursa found in birds. In vitro studies of these tissues were not possible due to the very few cells isolated by Hypaque-Ficoll gradient centrifugation. No gut associated lymphoid tissue or lymph nodes were found.

Separation Technique

Hypaque-Ficoll (ρ = 1.077) was used to isolate relatively pure lymphocyte preparations from heparinized whole blood or organ cell suspensions. White cell differential counts of fractionated and unfractionated blood are presented in Table 12 and illustrate the efficiency of the technique for isolating lymphocytes. Hypaque-Ficoll isolates routinely contained only about 5% granulocytic cells (predominately basophilic staining cells by May-Grunwald-Giemsa stain), and about 5% red blood cells. Approximately 2-3% of the granulocytes were phagocytic (assayed by collodial carbon uptake). Examination of the cells recovered from the interface and within the Hypaque-Ficoll gradient showed > 99% of the lymphocytes were present at the interface. A photomicrograph of a representative isolate is presented in Figure 7.

Culture Conditions

Various sera were tested to determine a suitable supplement with MEM for in vitro studies. Ten percent alligator, human, calf, fetal calf, and rabbit sera or all combinations of equimixtures (5% per serum)

Table 12

White Cell Differentials of Alligator Whole Blood and Hypaque-Ficoll Isolated Blood Cells

	Percent of Total[b]	
Cell Type[a]	Blood	Hypaque-Ficoll Isolated
Thrombocyte	4±2	0
Granulocyte	36±4	5±5
Lymphocyte	60±2	95±5

(a) Smears were made of whole blood and Hypaque-Ficoll isolates of individual samples and were May-Grunwald-Giemsa stained for quantitation purposes.
(b) Results are expressed as a percent of the total number of white blood cells counted.
(c) Each value represents the mean of determinations from 10 different alligator samples (> 3 determinations per samples) ± standard deviations.

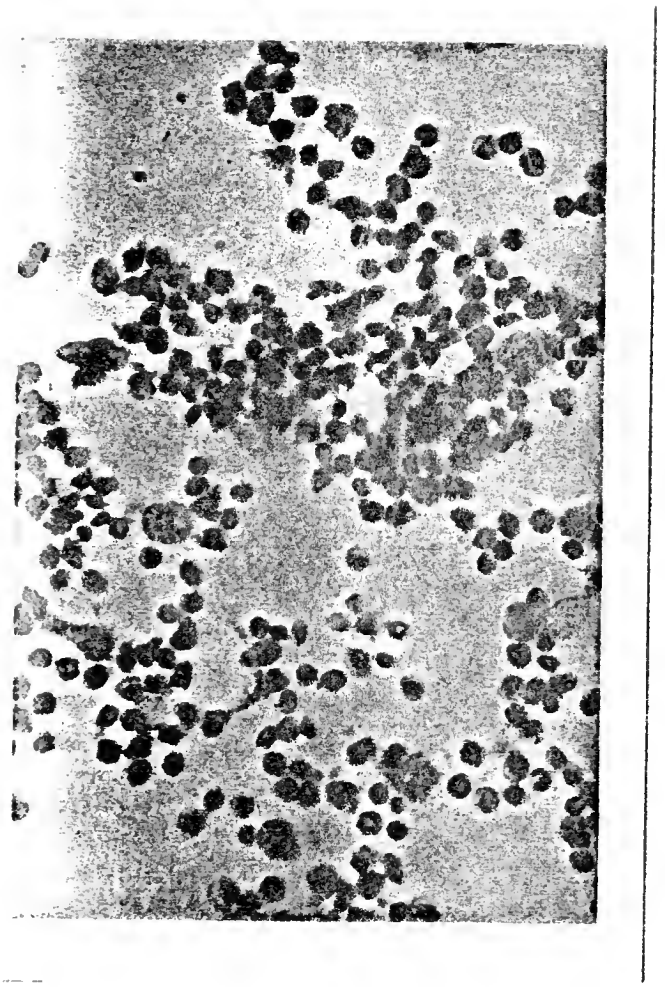


Figure 7. Photomicrograph of a representative Hypaque-Ficoll isolate of alligator peripheral blood. May-Grunwald-Giemsa stained. Magnification x 400.

of any two were tested. Only 10% alligator serum and 5% alligator-5% fetal calf serum supported in mitogen stimulation of lymphocytes. Although significant stimulation was obtained in cultures supplemented with an equimixture of alligator and fetal calf sera, stimulation indices were lower than those obtained from 10% alligator serum supplemented cultures and therefore 10% alligator serum was used routinely.

Not all alligator sera were supportive as a supplement and it was necessary to test new alligator supplement sources in mitogenic assays to determine their suitability. Tests of eight serum pools (> 10 individual bleedings per pool) obtained from 2-3 yr old alligators and four individual alligators > 10 yr old are presented in Table 13. Individual sera from older alligators were more effective than pools of sera from younger alligators. Since large volumes (200-500 ml) could be obtained from individual bleedings of 100-225 kg alligators, the older alligators were used exclusively as sources of serum in subsequent experiments.

Although statistically significant stimulation of alligator lymphocytes cultured with mitogens was obtained using 10% alligator serum supplemented MEM (0.117 M NaCl), severe cell clumping and loss of viability were noted when cells were suspended in the culture medium. To determine if the salt concentrations in the medium were appropriately matched to alligator serum levels, three alligator sera (obtained from individual bleedings) were analyzed by the Blood Chemistry Lab (Department of Pathology, J. Hillis Miller Health Center). Comparisons of the chemistry lab reports with the GIBCO MEM formulations revealed a reproducible difference in the NaCl concentrations. On the basis of this finding an experiment in which alligator peripheral blood lymphocytes

Table 13

PHA Responses of Alligator Peripheral Blood Lymphocytes Cultured with Different Alligator Serum Supplements

Supplement		Stimulation Index[a]
Pool[b]	A	1
	B	36
	C	45
	D	18
	E	18
	F	7
	G	5
	H	19
Alligator[c]	AA	78
	BB	1
	CC	90
	DD	100

(a) Triplicate cultures were incubated at 32°C with or without PHA (1 µl), pulsed on day 4 and harvested on day 5.
(b) Each supplement pool is from > 10 individual bleeds of 1-2 kg alligators 2-3 yrs old.
(c) Individual serum supplements are from bleeds of 100-225 kg alligators > 10 yrs old.

were stimulated with PHA in different MEM preparations containing various concentrations of NaCl was conducted. The results of this experiment are presented in Table 14. TCA precipitable counts of PHA-stimulated cells cultured in mammalian MEM were significantly increased ($p < 0.05$) over control counts. However, stimulation indices of cells cultured with 0.157 M or 0.177 M NaCl concentrations (0.040 M and 0.060 M extra NaCl respectively) were approximately three times greater than the stimulation index of cells cultured in mammalian MEM. Also cell clumping and loss of viability were no longer evident. Therefore, the NaCl concentration of MEM was routinely increased by 0.04 M to 0.157 M in all media used in subsequent in vitro studies with alligator lymphocytes.

To determine if optimal conditions for pulsing mammalian cultures with ^{3}H-thymidine (0.5-1.0 µCi/culture; 24 hr) were applicable for alligator lymphocyte cultures, the effects of ^{3}H-thymidine concentrations used per well and the length of the pulse were examined. The data presented in Tables 15 and 16 indicate that incubating the cultures with 0.5 µCi ^{3}H-thymidine for 24 hr prior to culture termination was optimal for pulsing alligator lymphocyte cultures.

Mitogenic Studies

Since large numbers of lymphocytes could be obtained from single bleedings (4-8 x 10^{8} lymphocytes from 40 ml of blood), large scale experiments were designed to determine the effects of 1) mitogen dose, 2) length of time in culture, and 3) temperature on the responses of peripheral blood lymphocytes to phytohemagglutinin (PHA), concanavalin A (Con A), lipopolysaccaride (LPS), pokeweed mitogen (PWM), and purified protein derivative (PPD).

Table 14

Effect of Sodium Chloride Concentration on Alligator Peripheral Blood Lymphocytes Cultured with PHA

NaCl Concentration[a]	Stimulation Index[b]
0.117 M[c]	47
0.137 M	85
0.157 M	142
0.177 M	145
0.197 M	59
0.217 M	1.0

(a) Final concentrations of NaCl in the culture medium.
(b) Cultures were incubated at 32°C with or without PHA (1 µl), pulsed on day 4 and harvested on day 5.
(c) The concentration of NaCl in mammalian MEM was calculated from the GIBCO formulation to be 0.117 M.

Table 15

Effect of ^{3}H-Thymidine Concentration on Alligator Peripheral Blood Lymphocytes Cultured with PHA

µCi ^{3}H/Culture	Stimulation Index[a]
0.05	30
0.1	60
0.25	66
0.5	71
1.0	71
2.0	67

(a) Cells were incubated at 32°C with or without PHA (1 µl), pulsed on day 4 and harvested on day 5.

Table 16

Effect of Incubation Time with ^{3}H-Thymidine on PHA Stimulated Alligator Peripheral Blood Lymphocytes

Length of Time with[a] ^{3}H-Thymidine (Hr)	Stimulation Index
24	108
48	119
72	112
96	110

(a) Cells were cultured at 32°C with or without PHA (1 µl). Five-tenths µCi of ^{3}H-thymidine was added at various intervals prior to the harvest of cultures. All cultures were terminated on day 5.

The results of one experiment designed to test the effects of temperature on responsiveness of alligator lymphocytes to PHA are presented in Figure 8. Cells were cultured with various doses of PHA at the temperatures indicated and the optimal dose and the length of time for maximum stimulation was determined at each temperature. The results indicate that the lower the temperature, the longer the time required for maximum stimulation (indicated in parenthesis). The response of cells cultured at 22°, 35°, 37°, and 40°C was significantly lower ($p < 0.01$) than in cells cultured at 27°, 30°, and 32°C. Although responses of cells incubated at 27°, 30°, and 32°C were not significantly different from each other ($p > 0.1$), the length of time required for optimal stimulation of cultures maintained at 32°C was shorter (five days) as compared with 27° and 30°C maintained cultures (seven days). The optimal mitogen dose was found to be the same at all temperatures. Typical responses to various mitogen doses and incubation times of alligator lymphocytes cultured with PHA and LPS at 32°C are presented in Figures 9 and 10 respectively. The response to PHA peaked sharply on day 5 and decreased slowly, whereas the peak response to LPS remained elevated after reaching an optimum on day 5. Similar experiments were performed with each of the other mitogens and the results can be summarized by stating that the optimal temperature tested was found to be 32°C, the length of time for maximum stimulation was five days and the optimal mitogen dose was the same at each temperature tested. Optimal doses per culture of LPS, PPD, PWM, PHA and Con A were 10 µg, 10 µl, 1 µl, and 20 µg respectively. It should be pointed out that responses of cells cultured with 20 µg of Con A varied in different experiments and was attributed to changes in the lot numbers of Con A used, as well as the

Figure 8. The effects of temperature on the responsiveness of alligator peripheral blood lymphocytes to PHA. Cultures were stimulated with 1 µl of PHA. Numbers within parentheses indicate the length of time (days) to maximum stimulation at the designated temperature.

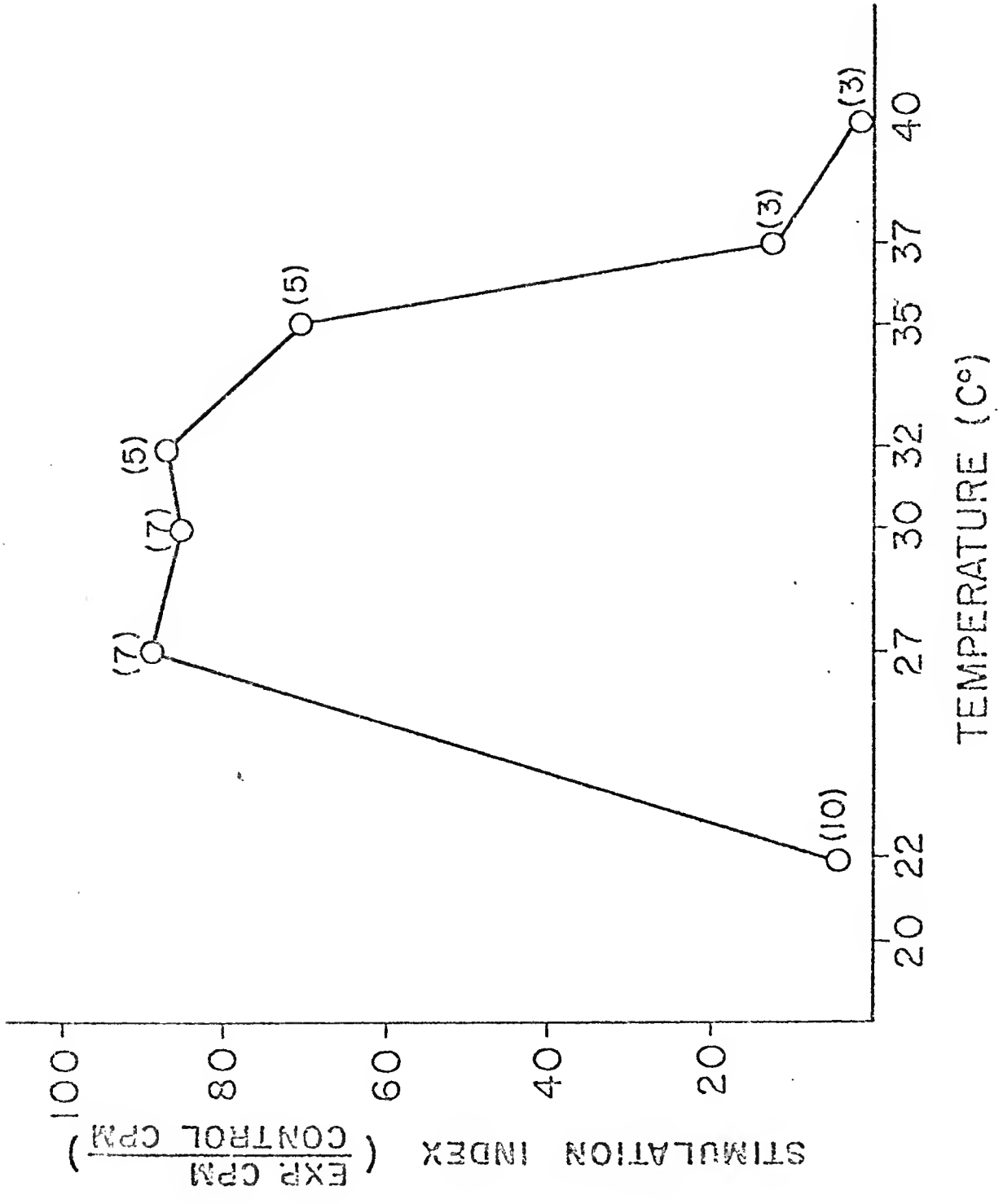

STIMULATION INDEX (EXP CPM / CONTROL CPM)
100
80
60
40
20
20
22
27
30
32
35
37
40
TEMPERATURE (C°)
(110)
(7)
(7)
(5)
(5)
(3)
(3)

Figure 9. Dose and time response of alligator peripheral blood lymphocytes cultured with PHA. All cultures were incubated at 32°C.

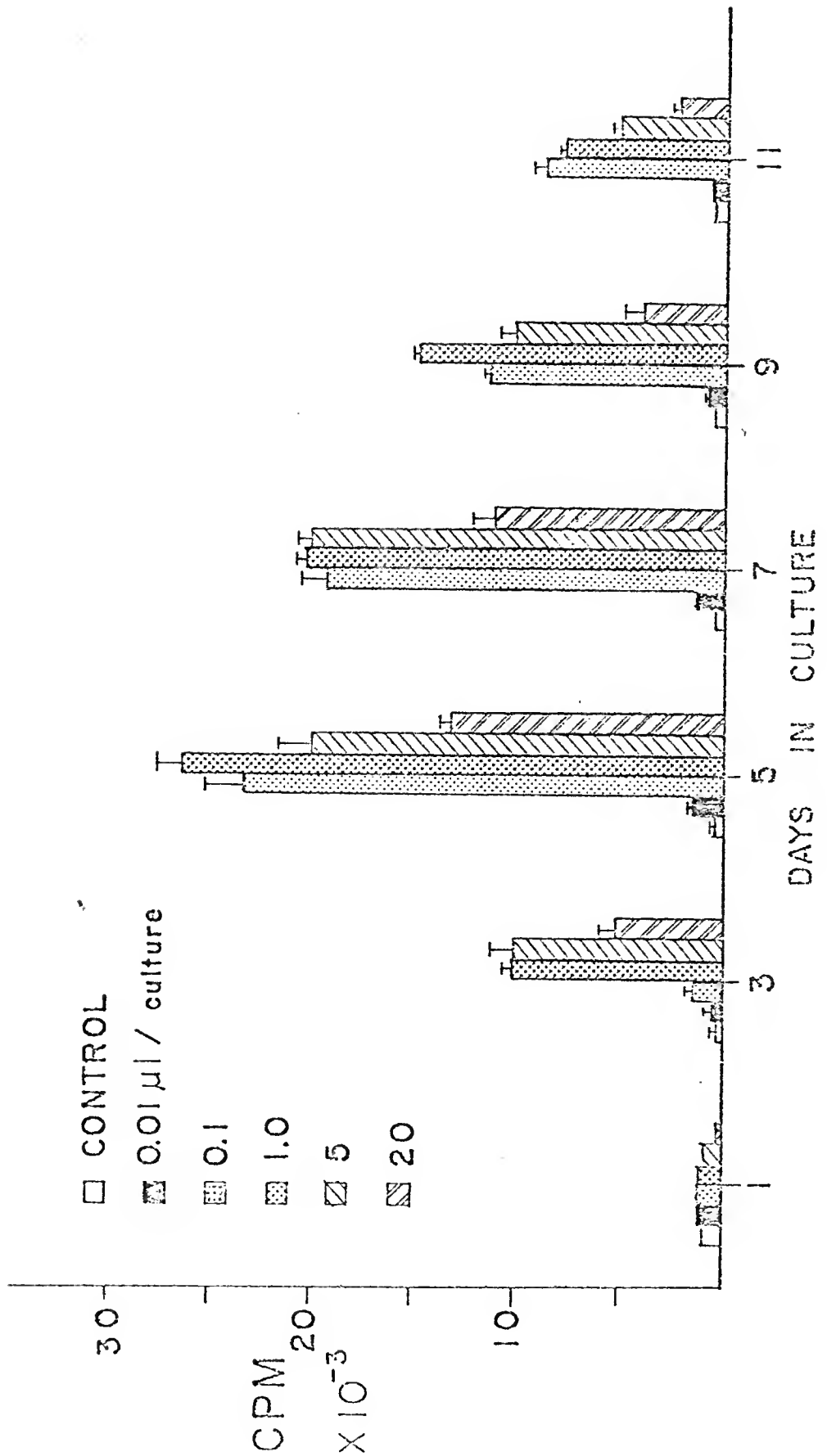
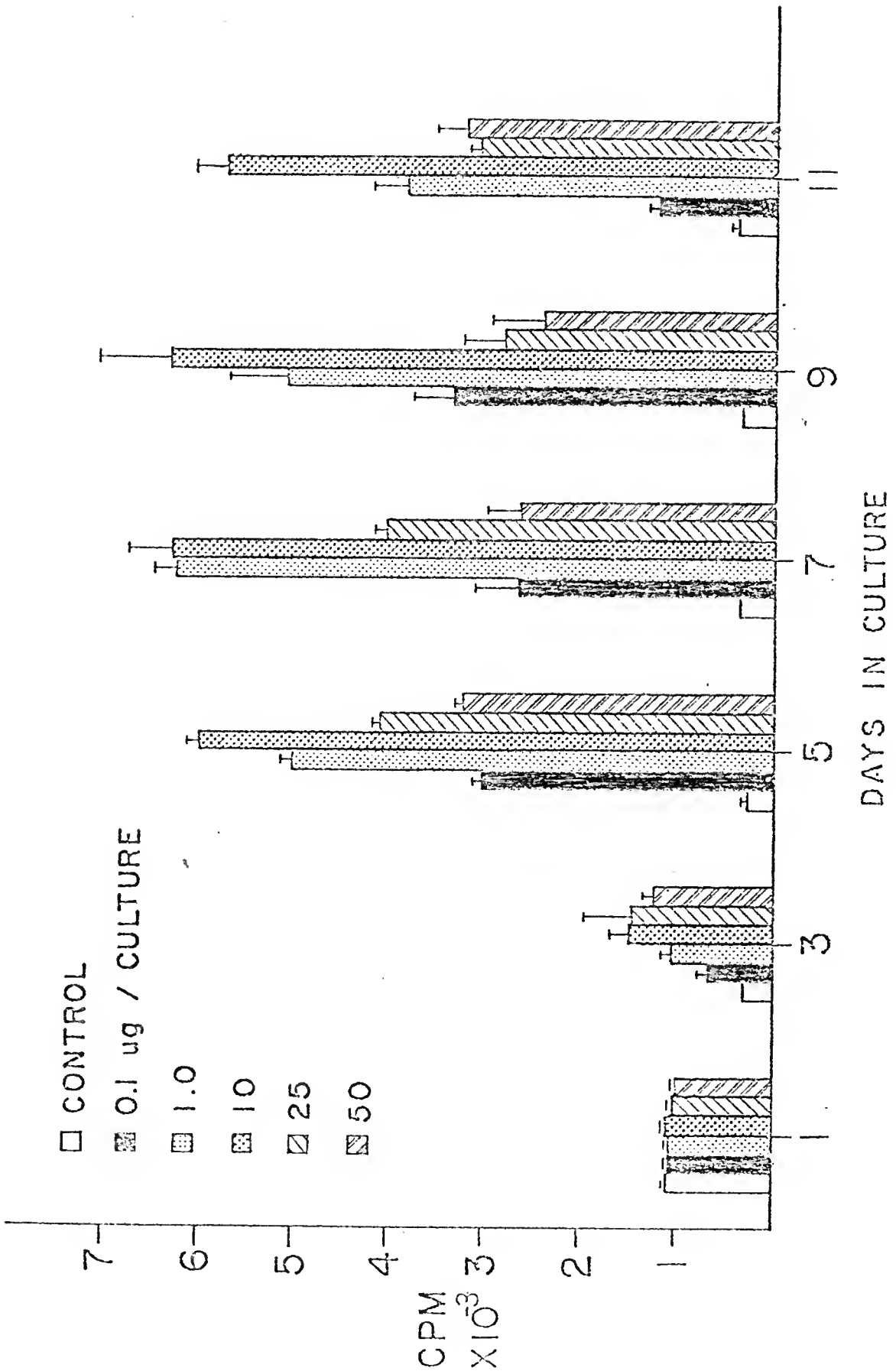

CONTROL
0.01 µl / culture
0.1
1.0
5
20
CPM X10⁻³
30
20
10
1
3
5
7
9
11
DAYS IN CULTURE

Figure 10. Dose and time response of alligator peripheral blood lymphocytes cultured with LPS. All cultures were incubated at 32°C.


CONTROL
0.1 ug / CULTURE
1.0
10
25
50
CPM X10^-3
7
6
5
4
3
2
1
1
3
5
7
9
11
DAYS IN CULTURE

length of time a mitogen solution was stored at 4°C. Responses to PHA or Con A were always significantly greater ($p < 0.01$) than responses to LPS, PWM, or PPD, with stimulation indices of PHA or Con A ranging from 40-250 and those for LPS, PWM, or PPD stimulated cultures between 1-25.

Assay for In Vitro Cellular Reactions

To determine if TCA precipitable counts were a valid measure of cellular events in culture, the number of labeled cells stimulated with various concentrations of LPS or PHA were correlated with the TCA precipitable counts in experiments (Chapter II). The results are presented in Figures 11 and 12 and indicate that both LPS- and PHA-stimulated cultures exhibit changes in TCA precipitable counts closely paralleling those changes in percent of labeled cells identified by autoradiography. Cells optimally stimulated with PHA (1 µl) were predominately in aggregates and looked like lymphoblasts (Figures 13a and 13b). Cells optimally stimulated with LPS (10 µg) were also morphologically characterized as blast-like but were not clumped (Figures 14a and 14b).

Comparison of Peripheral Blood and Splenic Lymphocyte Mitogen Responses

To assay whether mitogenic responses of peripheral blood lymphocytes were similar to the mitogenic responses of lymphocytes from other sources, cell suspensions were prepared from various alligator lymphoid tissues. Only the spleen cell suspension yielded a sufficient number of lymphocytes (isolated by Hypaque-Ficoll) to culture in a mitogen assay. The results obtained from mitogenic stimulations of peripheral blood and splenic lymphocytes are presented in Table 17. Optimal dose and time responses of both isolated lymphocyte populations were the same

Figure 11. Correlation of TCA precipitable counts with the number of autoradiography positive cells from PHA-stimulated alligator lymphocyte cultures. Cultures were incubated at 32°C, pulsed on day 4 and assayed on day 5.

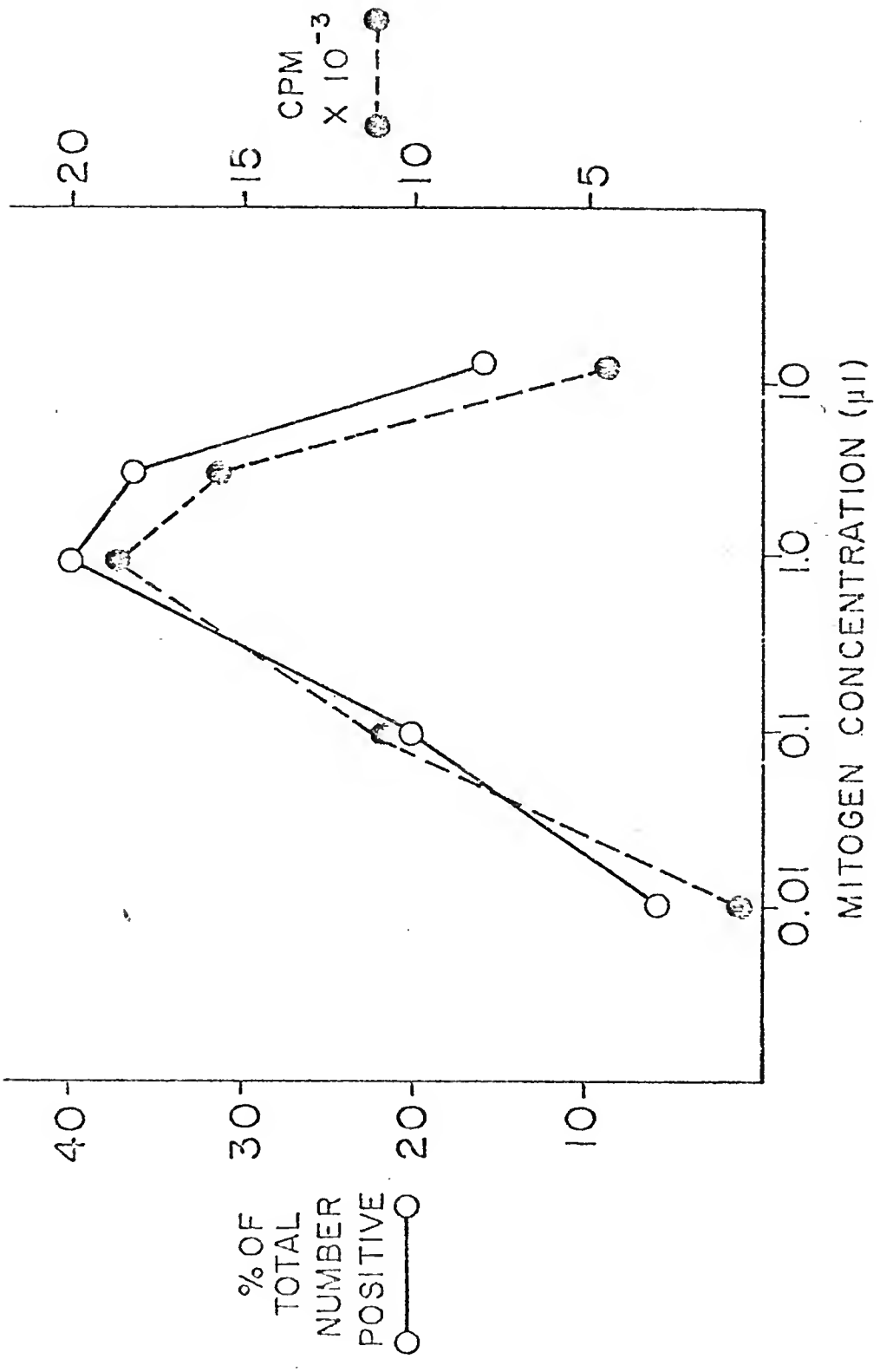

% OF TOTAL NUMBER POSITIVE
40
30
20
10
CPM X 10⁻³
20
15
10
5
0.01
0.1
1.0
10
MITOGEN CONCENTRATION (μl)

Figure 12. Correlation of TCA precipitable counts with the number of autoradiography positive cells from LPS-stimulated alligator lymphocyte cultures. Cultures were incubated at 32°C, pulsed on day 4 and assayed on day 5.

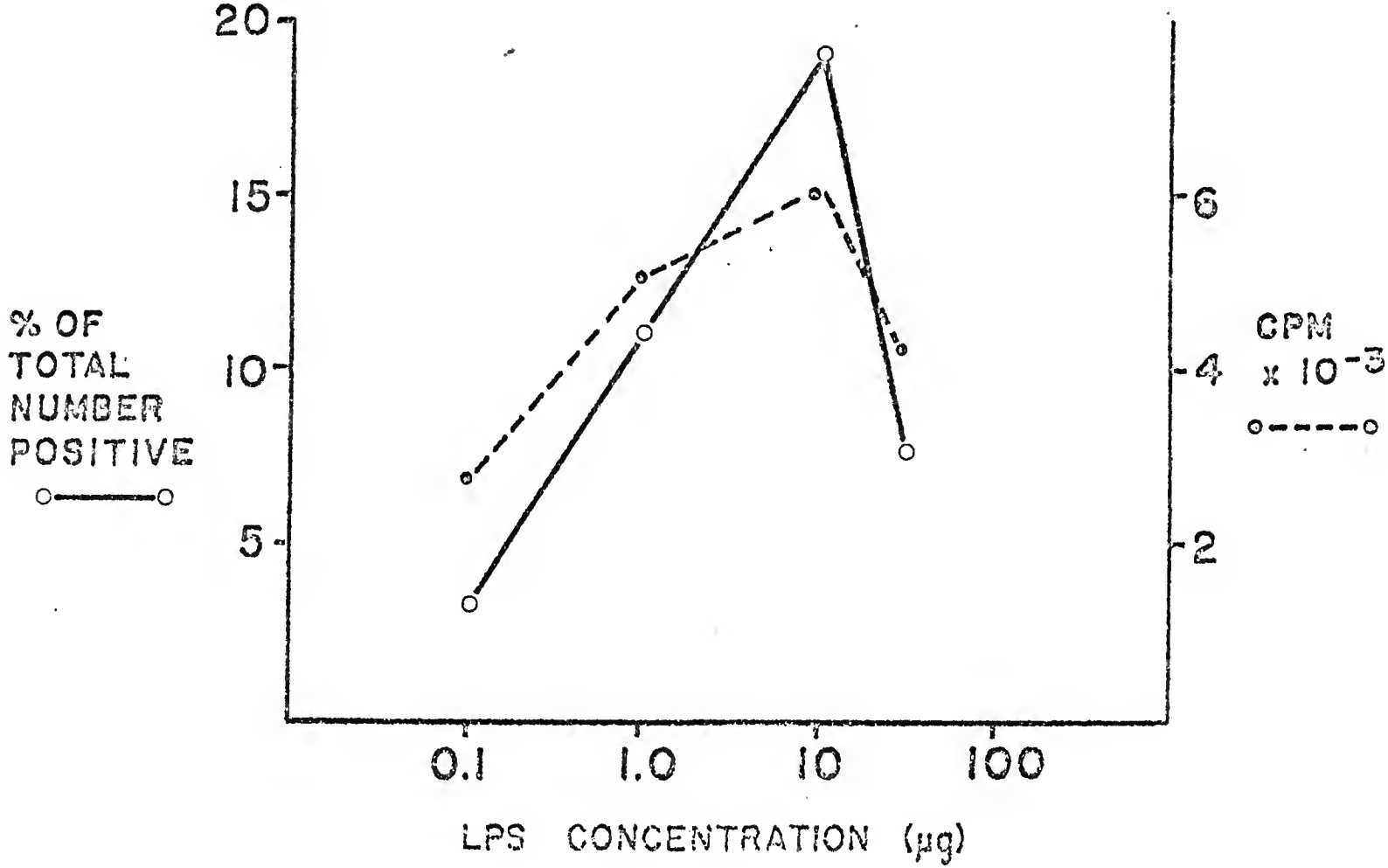
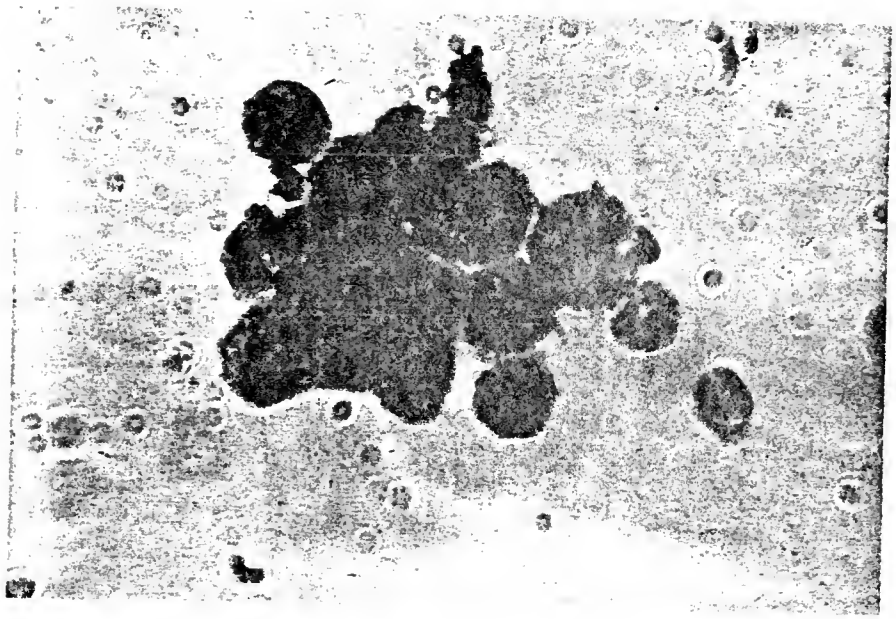

20
15
10
5
% OF TOTAL NUMBER POSITIVE
6
4
2
CPM x 10^{-3}
0.1
1.0
10
100
LPS CONCENTRATION (μg)

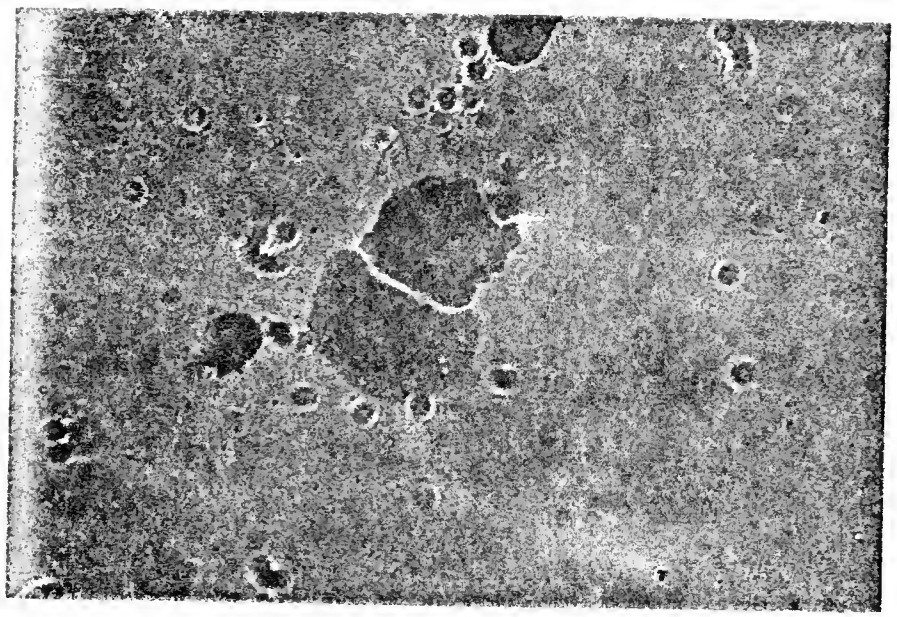
Figure 13. Photomicrograph of PHA-stimulated alligator peripheral blood lymphocytes. (a) Autoradiograph stained with toluidine blue. (b) May-Grunwald-Giemsa stained. Magnification x 1000.



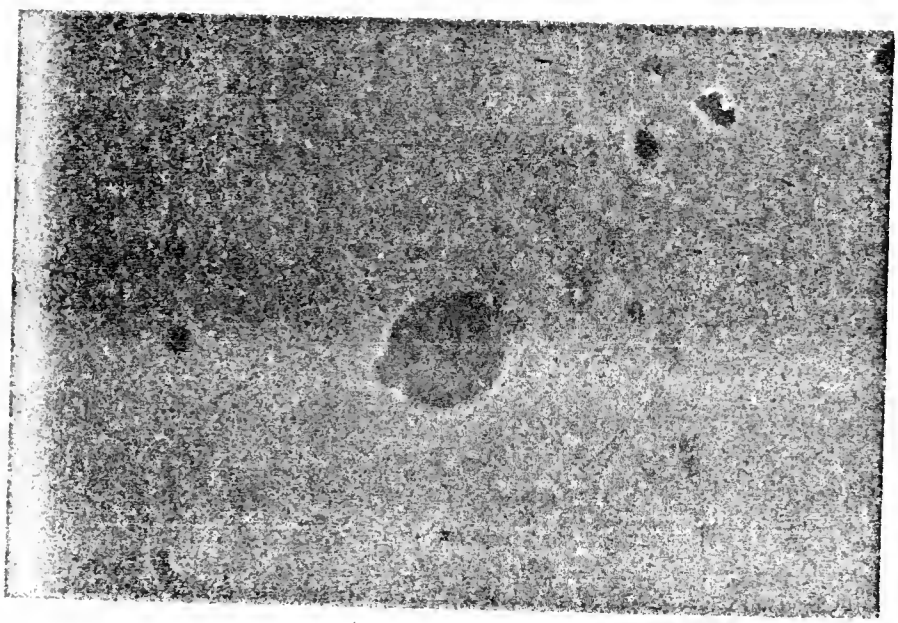
b

a

Figure 14. Photomicrograph of LPS-stimulated alligator peripheral blood lymphocytes. (a) Autoradiograph stained with toluidine blue. (b) May-Grunwald-Giemsa stained. Magnification x 1000.



b



a

Table 17

A Comparison of the Mitogen Responses of Alligator Blood and Splenic Lymphocytes

	Stimulation Index[a]	
Mitogen	Blood	Spleen
LPS (10 µg)	10	1
PWM (10 µg)	3	6
PPD (10 µg)	2	6
PHA (1 µl)	192	142
Con A (10 µg)	49	47

(a) Cell suspensions of blood and spleen were separated on Hypaque-Ficoll. Control and mitogen stimulated cultures of Hypaque-Ficoll isolates were incubated at 32°C, pulsed on day 4 and harvested on day 5.

for each mitogen, with the exception of the splenic lymphocyte response to LPS, in which case no response was observed. PHA and Con A responses in the two preparations were comparable. Responses to PWM and PPD were similar ($p > 0.1$) for splenic and peripheral blood lymphocytes.

Mixed Lymphocytes Cultures

Peripheral blood lymphocytes from different alligators were tested for their ability to respond in two-way mixed lymphocyte cultures (MLC's). Eight alligators were bled and the lymphocytes isolated by Hypaque-Ficoll. The results of all possible two way MLC's as well as PHA stimulation indices (from individual animals) are presented in Table 18. Optimal MLC responses were obtained on day 5 and remained high through day 7. There was a wide spectrum of responses, expressed as stimulation index, ranging from 1 (no response) to 12.5. Although the response to PHA was significantly lower ($p < 0.05$) in one of the alligators (alligator number 4) than the other seven, there was no correlation between a low response to PHA and the ability to respond in MLC.

The Combined Mitogen Effects on Lymphocyte Stimulation

The responses of alligator peripheral blood lymphocytes to various combinations of the mitogens were assayed to determine if different combinations would give additive, synergistic or antagonistic effects. The data from one experiment are presented in Table 19. Although in some cases it was difficult to determine if the combined effects were additive, synergistic or antagonistic when compared to the results from cultures stimulated with only one mitogen, the results from culturing lymphocytes with combinations of LPS + PHA, PWM + PHA and PWM + Con A

Table 18

Mixed Lymphocyte Cultures of Alligator Peripheral Blood Lymphocytes

	Stimulation Index[a]								
	Mixed Lymphocyte Culture								
Alligator	1	2	3	4	10	41	43	77	PHA Response[b]
1	-	1	12.5	2	2	10	6.5	13	167
2		-	3.4	3.3	1	8.5	4	10	143
3			-	5.5	3.7	6.5	5	8	123
4				-	1	3	2.6	3.9	76
10					-	3.5	4	3.4	157
41						-	4.5	4.5	207
43							-	8.5	145
77								-	172

(a) Cultures were incubated at 32°C, pulsed on day 4 and harvested on day 5.
(b) Cells were stimulated with 1 µl of PHA.

Table 19

Combined Effects of Mitogens on Alligator Peripheral Blood Lymphocytes

Mitogen(s)	Stimulation Index[a]
LPS (10 µg)	6.7
PWM (10 µl)	3.3
PHA (1 µl)	58
Con A (20 µg)	25
LPS + PWM[b]	17
PHA + Con A	55
LPS + PHA	142
LPS + Con A	31
PHA + PWM	22
Con A + PWM	7

(a) Cultures were incubated at 32°C, pulsed on day 4 and harvested on day 5.
(b) Optimal concentrations of the mitogens were used in the combined stimulations.

clearly indicated that there were at least two different effects on the stimulation of lymphocytes by the different combinations of mitogens. The effect of LPS + PHA was synergistic since the response was ∿ 2.5 times higher ($p < 0.01$) than the PHA response alone, ∿ 20 times ($p < 0.001$) higher than the LPS response and greater than a twofold increase of the sum of the LPS and PHA responses. The effect of PWM + PHA or PWM + Con A was antagonistic, in that the responses were ∿ 0.6-0.7 times lower ($p < o.o5$) than the PHA or Con A responses. The results from LPS + PWM, PHA + Con A and LPS + Con A stimulations were not sufficiently conclusive to determine if the effects were additive or synergistic.

Effect of Environmental Temperature on LPS Responsiveness

During the winter months a decrease in LPS responsiveness of alligator peripheral blood lymphocytes was observed. A subsequent return of responsiveness to LPS occurred with the arrival of spring weather. The data compiled from studies of one alligator from Nov. 20, 1975,to Feb. 27, 1976,are presented in Figure 15. Since the animals were housed outdoors and the winter was unusually cold, it was hypothesized that such colder environmental temperatures may have effected the circulating population of LPS-responsive lymphocytes. To test this hypothesis two alligators were housed indoors at 16°C for an extended period of time and the mitogen responsiveness of their peripheral blood lymphocytes (cultured at 32°C) was monitored periodically. The results presented in Table 20 indicate that environmental temperature did significantly effect the LPS-responsive population since a LPS response was not detected in either alligator after 36 days at 16°C. Although the PHA response appeared to drop also it was nonetheless present when the

Figure 15. A chronological study during the winter months of alligator peripheral blood lymphocytes. Beginning and ending dates are indicated within the parentheses on the abscissa. Cells were cultured at 32°C, pulsed on day 4 and harvested on day 5. LPS and PHA concentrations were 10 µg and 1 µl, respectively. S.I. = Stimulation Index.

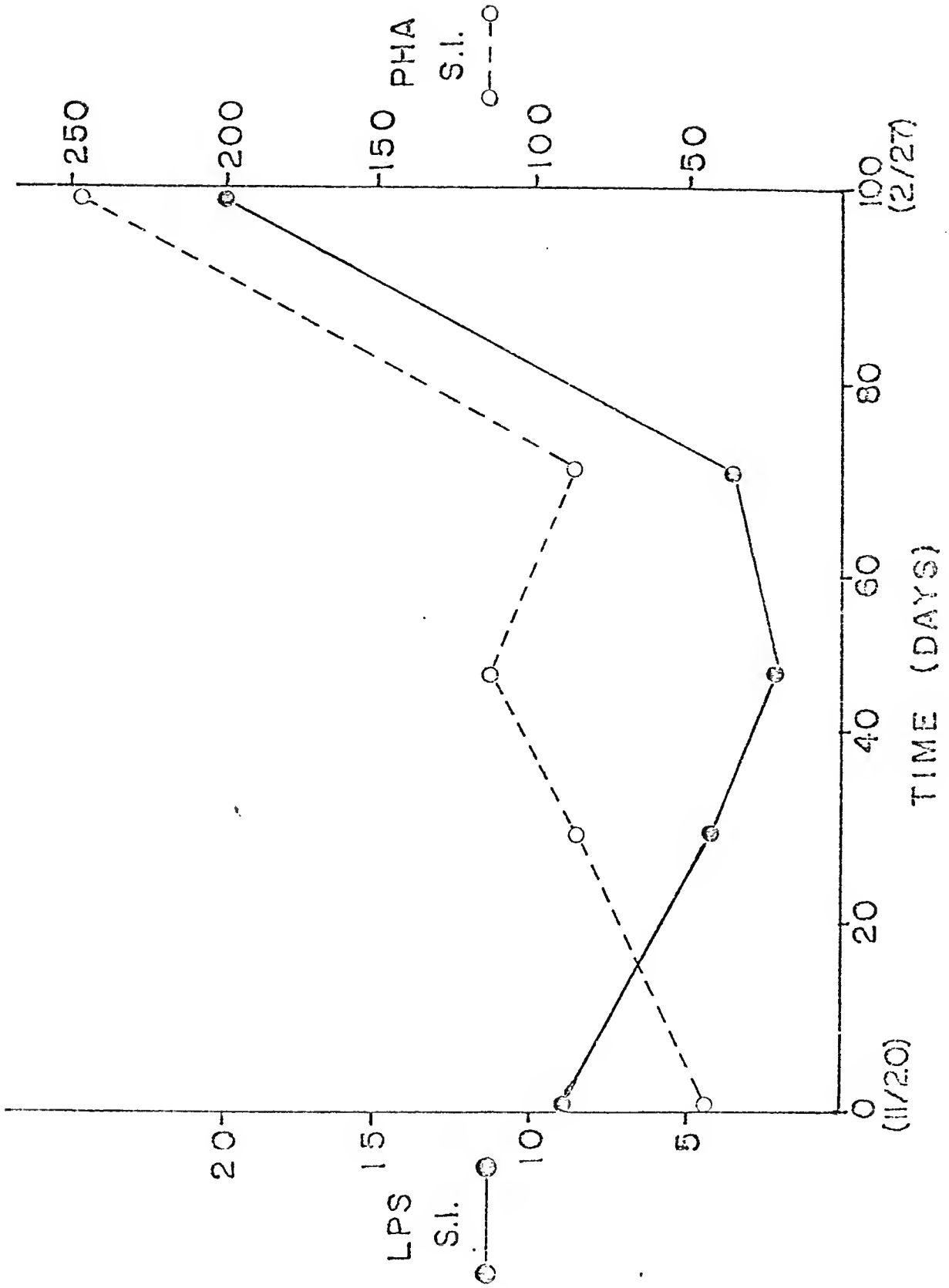

250
200
150
100
50
PHA
S.I.
100
(2/27)
80
60
40
20
0
(11/20)
TIME (DAYS)
20
15
10
5
LPS
S.I.

Table 20

Mitogen Responses of Peripheral Blood Lymphocytes from Alligators Maintained at 16°C

Alligator	Mitogen[a]	Time at 16°C			
		0	7	16	36
X	LPS (10 µg)	6.5	4.4	1	1
	PHA (1 µl)	250	100	30	59
77	LPS (10 µg)	4.9	10	5.8	1
	PHA (1 µl)	83	132	75	35

(a) Cultures were incubated at 32°C, pulsed on day 4 and harvested on day 5.

LPS response had disappeared. Upon returning alligator X to an outdoor environment for three months (May, June and July) the LPS responsive cells were again detectable (S.I.'s for LPS and PHA were 5 and 72 respectively).

Evidence for Two Populations of Alligator Lymphocytes

Differences in the magnitude of the stimulation obtained with the different mitogens as well as variations in the combined effects of different mitogens suggested that there may be at least two types of lymphocytes responding in mitogenic assays. The following results are from experiments designed to demonstrate that at least two different lymphocyte populations are present in cell suspensions isolated by Hypaque-Ficoll from the peripheral blood of alligators. To limit the number of variables tested, PHA and LPS were the primary mitogens used to follow mitogen responses after various cell manipulations.

Experiments were designed to determine if different populations of lymphocytes could be separated on the basis of their adherence or nonadherence to nylon wool or glass wool. Cells passed through columns filled with nylon wool or glass wool could be separated into two fractions; nonadherent and adherent fractions. These fractions were then cultured with various mitogens to determine if they exhibited differences in responses to mitogen stimulation. Nylon wool columns proved ineffective in that there was no difference between the nonadherent and adherent fractions in response to PHA, LPS, Con A, or PWM stimulation. However cell fractions obtained from glass wool columns did show different mitogen responses. In three experiments the nonadherent cells reproducibly exhibited significantly ($p < 0.05$) higher PHA (and Con A)

responses than unfractionated or adherent cells and very low or no LPS (and PWM) responses. The response of the adherent cells to PHA was not different from the responses of unfractionated cells ($p > 0.1$), but showed a significant ($p < 0.01$) increase in response to LPS over unfractionated cells.

It was hypothesized that there was an enriched population of cells responsive to PHA in the nonadherent fraction and an enriched population of cells responsive to LPS in the adherent fraction. It was further speculated that the response to PHA in the adherent cell fraction could be accounted for by nonspecific trapping of a nonadherent cell population within the glass wool fibers which was eluted along with the cell population responsive to LPS only after mechanically disrupting the glass wool. To determine if the cell population responsive to PHA could be depleted from the LPS-responsive cells in the adherent fraction and if the PHA-responsive cells in the nonadherent fraction could be further enriched, the nonadherent and adherent fractions were recycled through glass wool columns. Figure 16 is a diagram of the procedure used and the results from one such experiment are presented in Table 21. A higher response to PHA was obtained in the cell fraction which was nonadherent to either column (NA NA) and no response to LPS was detectable. The cell fraction adherent to both columns (A A) exhibited a higher LPS response ($p < 0.01$) than the unfractionated or adherent populations. However the response to PHA was only slightly lower ($p > 0.1$) than the original unfractionated population. In fact the response to PHA of the initial adherent fraction (A) in this particular experiment was significantly higher ($p < 0.05$) than the unfractionated population (0).

In additional experiments similar results were obtained. Responses to LPS were undetectable in the NA NA fractions and the responses to

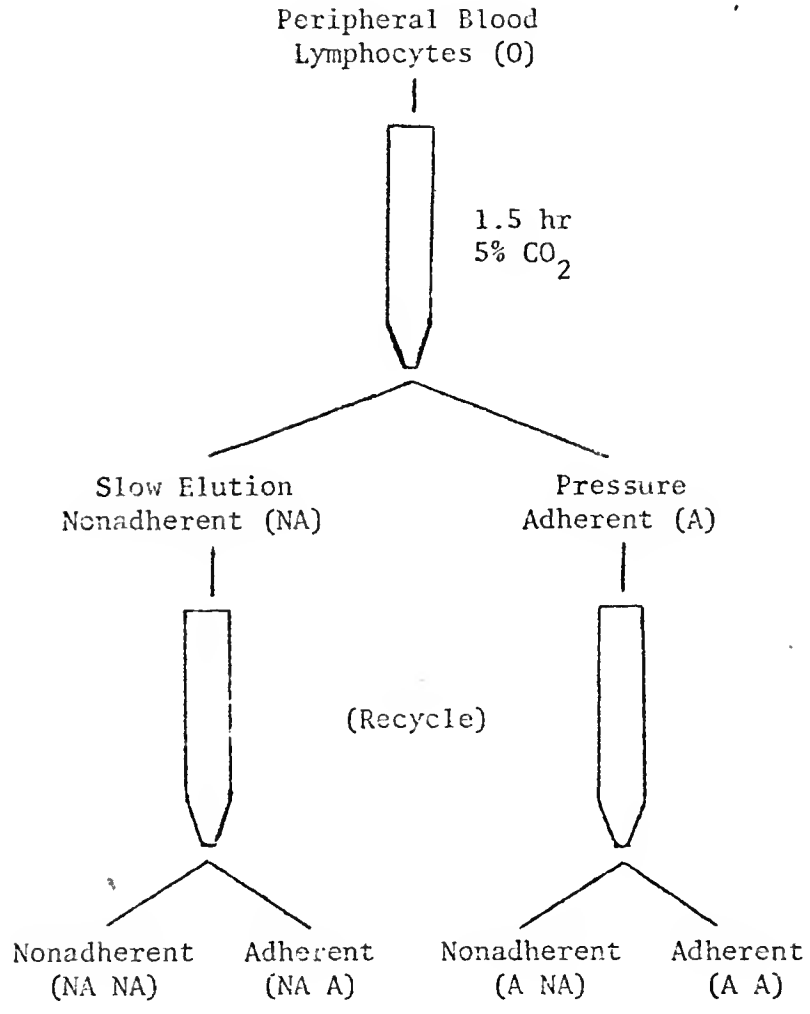


Figure 16. Diagram of glass wool fractionation procedures. One hundred million alligator peripheral blood lymphocytes or cells from eluted fractions were loaded onto the designated columns.

Table 21

Mitogen Responses of Cell Populations
Fractionated on Glass Wool

	Stimulation Index[a]						
	O[b]	NA	A	NA NA	NA A	A NA	A A
LPS (10 µg)	1.5	1.4	6.4	1.3	1.0	3.1	40
PHA (1 µg)	87	248	188	463	210	291	72

(a) Triplicate cultures were incubated at 32°C, pulsed on day 4 and harvested on day 5.
(b) See Figure 16 for an explanation of abbreviations.

PHA were significantly higher than the NA fractions or unfractionated cells. The LPS responsiveness in the A A fractions were significantly higher than the responses of the A fractions or unfractionated cells. The NA NA fraction routinely represented 30-50% of the original pupulation and the A A fraction 10-20%. The small percentage of granulocytes (see Table 12) present in the original cell population isolated from Hypaque-Ficoll were adherent to the glass wool and were not eluted by the procedures used in these experiments. The main cell type eluted (nonadherent or adherent) was a lymphocyte.

The response to PHA in the A A fractions were not reduced to significantly lower levels than the unfractionated cell response, so that from these experiments it was not possible to determine if the responses to LPS and PHA in the A A fractions were actually the responses of two different cell types (both adherent to glass wool or one adherent and one nonspecifically trapped) or whether there was only one cell type capable of responding to both mitogens. However the responsiveness of the NA A fractions only to PHA and not to LPS indicates that indeed there may be nonspecific trapping in the glass wool of a PHA-responsive cell population. It should be pointed out that the mitogen response of the NA A fraction could also be explained by an adherent cell population responsive only to PHA which was present in the NA fraction due to overloading the glass wool in the initial column fractionation.

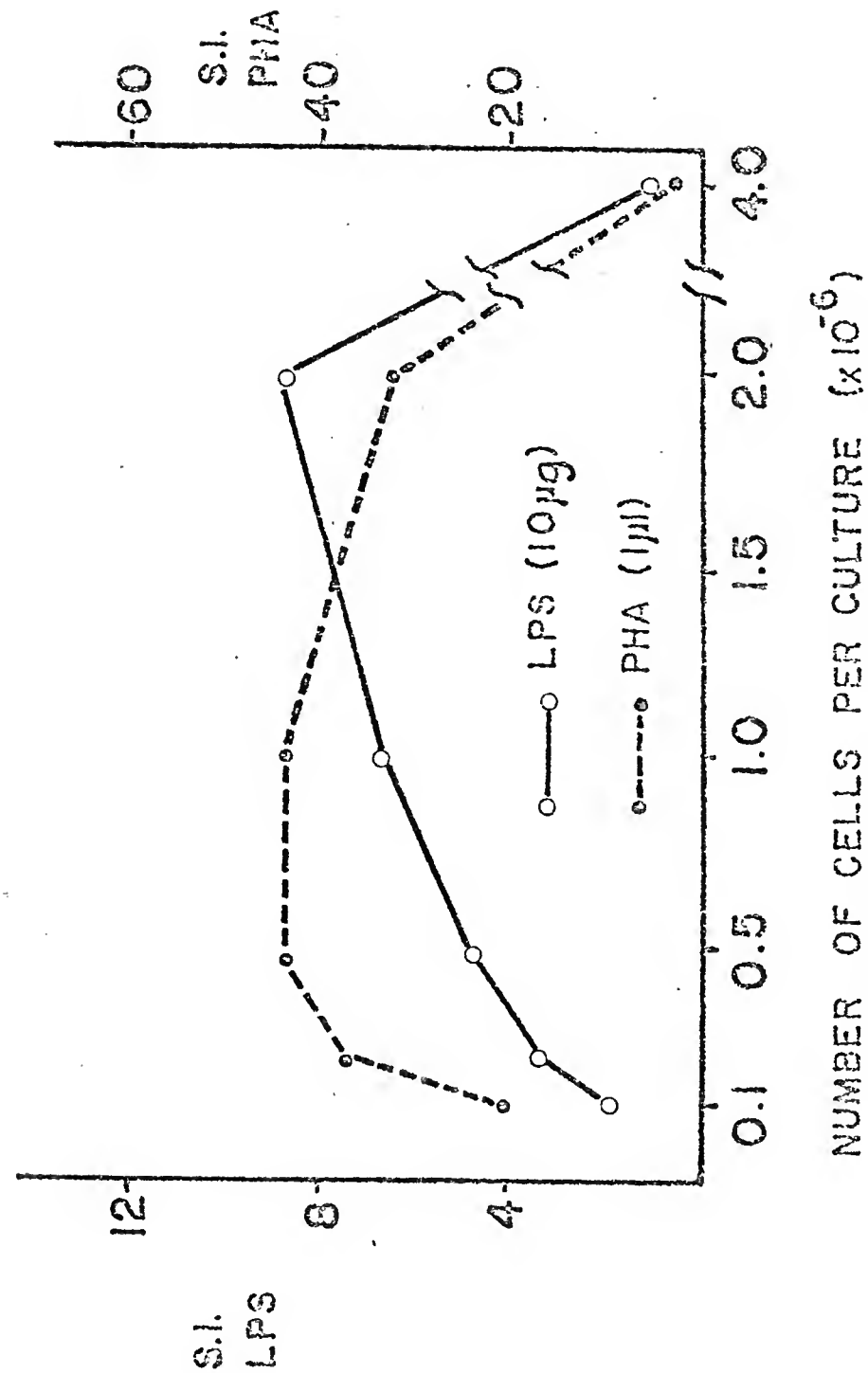
The significantly higher responses to PHA in the NA NA fraction and LPS in the A A fraction could perhaps be explained on the basis of a removal of suppressor cells (possibly the granulocytes suppressive for a particular mitogenic response), rather than simply an enrichment of a

cell type capable of responding to a particular mitogen. Experiments were designed to determine if the mitogen responses of isolated fractions could be depressed by co-culturing, in various ratios of cell numbers, mixtures of NA NA or A A fractions with either the unfractionated population (0) or each of the fractionated populations. The results from three separate experiments (not shown) did not lend evidence to the idea that suppressor cells were removed by passage through the columns. Decreases in the PHA or LPS responsiveness of the NA NA or A A fractions co-cultured with various other fractionated or unfractionated cell populations could be accounted for by effects of the NA NA or A A populations.

Further evidence which supports the theory that certain cell types were enriched by passage through a glass wool column was obtained from experiments in which unfractionated cells were cultured at various cell concentrations with PHA and LPS. On the basis of the glass wool fractionation data the unfractionated cell population with an undetectable LPS response could have contained cells capable of responding to LPS which were not detectable in the assay due to their low number. The results presented in Figure 17 indicate that by increasing the number of cells per culture, a significant increase ($p < 0.05$) in the response to LPS was obtained. A similar effect was seen with PHA over a narrower range of cell concentrations.

To summarize the results from glass wool fractionation studies, the data indicate that at least two lymphocyte cell populations are present in the peripheral blood of alligators: 1) a lymphocyte population which is nonadherent to glass wool and is responsive to PHA and 2) a lymphocyte population which is adherent to glass wool and is responsive to LPS (and possibley to PHA).

Figure 17. Effects of increasing the cell density in mitogen stimulated cultures of alligator peripheral blood lymphocytes. Cultures were incubated at 32°C, pulsed on day 4 and harvested on day 5. S.I. = Stimulation Index.


S.I. LPS
12
8
4
S.I. PHA
60
40
20
0.1
0.5
1.0
1.5
2.0
4.0
LPS (10μg)
PHA (1μl)
NUMBER OF CELLS PER CULTURE (x10⁻⁶)

To determine if different populations of lymphocytes could be isolated on the basis of differences in cell surface antigens, peripheral blood lymphocytes were treated with different antisera plus complement and the surviving cells cultured *in vitro* with various mitogens. Efforts to produce a rabbit anti-alligator brain with specific reactivity to a subpopulation of alligator lymphocytes were unsuccessful, in that 100% of the lymphocytes treated with the immune sera plus guinea pig complement were killed (as adjudged by trypan blue exclusion and cell recoveries). Adsorption of the antisera with alligator red blood cells removed all of the reactivity (∿ 100% of the cells were recovered as viable and had unaltered responses to mitogens). However, similar cytotoxic experiments utilizing a rabbit anti-alligator immunoglobulin plus complement were successful. The results of two experiments presented in Table 22 show that a population of lymphocytes responsive to LPS was depleted by anti-immunoglobulin plus complement and the PHA response remained intact. To determine if any residual responsiveness to LPS could be detected, the surviving cells in experiment 1 were cultured with the mitogens at a higher cell density. The rationale for this approach was based upon the previously mentioned effects of cell density. An increase in the number of cultured cells was shown to yield a response to LPS (see Figure 17). Only cells treated with normal rabbit serum plus complement showed an increase in responsiveness to LPS. As an additional assay to determine if cells responsive to LPS were being depleted by the cytotoxic treatment with anti-immunoglobulin plus complement, the synergistic action of LPS plus PHA (see Table 19) was measured. As shown in experiment 1, the synergistic effect was ablated only by the anti-immunoglobulin plus complement treatment. It should be

Table 22

The Effects of Cytotoxic Treatment with Rabbit Anti-Alligator Immunoglobulin on the Mitogen Responsiveness of Alligator Peripheral Blood Lymphocytes

Experiment	Treatment[b]	% Recovered[c]	Cells/Culture	Stiumlation Index[a]		
				LPS (10 µg)	PHA (1 µg)	LPS+PHA
1	NRS	94	$0.5x10^6$	5	41	84
			$2x10^6$	11	34	101
	Anti-Ig	71	$0.5x10^6$	1	45	34
			$2x10^6$	1	40	36
2	NRS	97	$0.5x10^6$	9	98	ND[d]
	Anti-Ig	86	$0.5x10^6$	2.5	75	ND

(a) Cultures were incubated at 32°C, pulsed on day 4 and harvested on day 5.
(b) Twenty percent rabbit serum (normal rabbit serum [NRS] or rabbit anti-immunoglobulin [Anti-Ig] plus 10% guinea pig complement was used to treat the cells.
(c) Viability was determined by trypan blue exclusion. The number of recovered viable cells was expressed as a percent of the original number of viable cells.
(d) ND = not done.

pointed out that the response to LPS was not always totally depleted, as shown in experiment 2. Possible reasons for such differences in anti-immunoglobulin plus complement treatments will be discussed in a later section.

Ten to 15% of the total number of lymphocytes were killed by anti-immunoglobulin plus complement treatment. However < 1% of the total number of lymphocytes were membrane immunoglobulin positive as measured by an indirect membrane immunofluorescence technique. Differences in the results obtained from the two techniques will be discussed in a later section.

Results from the cytotoxic experiments described above indicated that killing the cells with surface immunoglobulin determinants depletes the response to LPS. Another approach used to selectively remove cells bearing surface immunoglobulin, without complement mediated killing, was attempted. Cells were passed through cellular immunoadsorbents conjugated with either normal rabbit serum or rabbit anti-alligator immunoglobulin. Results from one experiment are presented in Table 23. Cells which passed through the anti-immunoglobulin immunoadsorbent column were stimulated only by PHA and not by LPS (or PWM). Attempts to elute the retained cells using whole alligator serum (i.e. antigenic competition) were not successful due to much reduced flow rates.
In conclusion, these results indicated that removal of surface immunoglobulin bearing cells depleted the response to LPS and left intact the population of cells capable of responding to PHA.

To determine if PHA- and LPS-stimulated cultures expressed different cellular characteristics in vitro, the number of cells producing immunoglobulin (cells containing immunoglobulin in their cytoplasm) were

Table 23

Depletion of LPS and PWM Responsiveness
in Alligator Peripheral Blood Lymphocytes Passed
Through an Anti-Immunoglobulin Immunoadsorbent

Treatment[a]	% Recovered[b]	Stimulation Index[c]		
		LPS	PHA	PWM
Unfractionated	-	5	74	9.8
NRS Immunoadsorbent	100	5.5	80	12.5
Anti-Ig Immunoadsorbent	67	1	71	1

(a) Immunoadsorbents were prepared from 40% saturated ammonium sulfate precipitates of rabbit anti-alligator immunoglobulin (Anti-Ig) or normal rabbit serum (NRS) coupled to Sephadex G-200.
(b) The number of cells recovered is expressed as a percent of the total number of cells applied to the immunoadsorbent column.
(c) Cultures were incubated at 32°C, pulsed on day 4 and harvested on day 5. Mitogen concentrations were 10 µg, 1 µl and 10 µl for LPS, PHA, and PWM respectively.

quantitated by indirect immunofluorescence. The results are presented in Table 24. Very few positive cells were seen in cell preparations (Hypaque-Ficoll isolates) before culturing. There was an increase in the number of immunoglobulin containing cells in PHA-stimulated cultures as compared to cells which were not cultured. However a similar increase was also seen in unstimulated cultured controls as well, and was considered to be a nonspecific increase due to culture conditions. On the other hand LPS-stimulated cultures showed a significant ($p < 0.01$) increase in the number of immunoglobulin positive cells over uncultured control, cultured control and PHA-stimulated cultures indicating that LPS stimulates immunoglobulin production in alligator lymphocytes as it does in mouse lymphocytes.

In Vitro Studies on Antibody Producing Cells

Due to the limited number of alligators available and the necessity of maintaining "normal" alligators as blood donors for *in vitro* experiments, it was not possible to study *in vivo* immune responses. However, one preliminary experiment was performed to determine if peripheral white blood cells isolated from Hypaque-Ficoll were immunocompetent in a primary *in vitro* immunization assay (Mishell-Dutton type cultures). Control (without SRBC) or immunized (with SRBC) cultures were maintained at 32°C and assayed for the number of cells producing antibody to SRBC's after various periods of incubation. The results are presented in Table 25. Only cells from immunized cultures contained plaque-forming cells. The peak response occured after seven days of culture (two days later than the optimal time for mitogenic stimulation). It should be pointed out that a decrease in the number of plaque-forming cells on day 10 may

Table 24

Cytoplasmic Immunofluorescence Studies of Uncultured and Cultured Alligator Peripheral Blood Lymphocytes

Cells[a]	% Positive[b]	
	NRS	Anti-Ig
Uncultured Control	0	0.1-0.3
Cultured Control	0	0.5-1.5
Cultured with PHA (1 µl)	0	0.5-1.0
Cultured with LPS (10 µg)	0	7-10

(a) Cultured cells were incubated with or without mitogens at 32°C for five days. Cytocentrifuged slides were prepared of cells obtained from cultures or Hypaque-Ficoll isolates of uncultured cells and were stained for cytoplasmic immunoglobulin.
(b) The number of positive staining cells is expressed as a percent of the total number of white cells counted. The "sandwich" stain was normal rabbit serum (NRS) or rabbit anti-alligator immunoglobulin (Anti-Ig) and the second stain was fluorescein conjugated goat anti-rabbit IgG.

Table 25

Primary In Vitro Immunization with Sheep Red Blood Cells of Alligator Peripheral Blood Lymphocytes

Culture[a]	Days in Culture	PFC/Culture	% Recovered[b]	% Viable[c]	% Rosettes[d]
Control	5	0	18	96	0
Immunized		0	34	93	33
Control	7	0	14	80	0
Immunized		908	90	91	29
Control	10	0	16	96	0
Immunized		19	94	54	28

(a) Trilpicate cultures were incubated at 32°C.
(b) The total number of white cells recovered at the end of the culture period is expressed as a percent of the initial number of white cells.
(c) The number of viable cells is expressed as a percent of the total number of white cells recovered. Viability was determined by trypan blue exclusion.
(d)The number of white cells rosetting with SRBC's is expressed as a percent of the total number of white cells.

have been due to a decrease in viability as a result of a nutrient depletion(s) in the culture medium (the pH was lower on day 10). There was an initial decrease in the number of cultured cells in both control and immunized cultures followed by an increase in the number of cells recovered from immunized cultures and an unchanged residual population in control cultures.

A high percentage ($\sim$ 30%) of the cells recovered from immunized cultures were observed to form rosettes with SRBC. These rosettes persisted (i.e. not lysed) through the Jerne assay and therefore probably did not represent antibody-producing cells.

To explore the possibilities that the rosetted cells recovered from immunized cultures were not antigen induced but rather represented either a population of cells normally present in the peripheral blood of alligator capable of rosetting SRBC's or cells which were generated by the culture conditions, irrespective of the presence of SRBC's, the following experiment was done. The alligator used as a blood donor was bled again at the time of culture termination and the blood separated on Hypaque-Ficoll. Cells recovered from control cultures (no SRBC's present) and immunized cultures (SRBC's present) as well as Hypaque-Ficoll isolated, uncultured cells were each tested for their ability to form rosettes with SRBC's. No rosettes were observed in cells recovered from control cultures and < 1% of the total number of uncultured lymphocytes isolated from Hypaque-Ficoll formed rosettes, whereas 31% of the cells recovered from immunized cultured were rosetted. These findings indicate that the high number of rosetted cells in immunized cultures may have resulted from antigenic stimulation.

Discussion

Culture Conditions

As with any initial study involving *in vitro* lymphocyte culture, it was important to investigate several variables in order to define optimal culture conditions. The data clearly demonstrated the necessity of establishing culture conditions optimal for the alligator rather than merely employing mammalian criteria of culture media and incubation temperature. Although mammalian MEM significantly supported stimulation of cells cultured with PHA, optimal stimulation was approximately three times higher when extra sodium chloride was added. Similarly, although cultures incubated at 37°C gave measurable stimulation indices with PHA stimulation, an assumption that a mammalian temperature optimum was optimal for alligator cultures would have been erroneous in that the temperature optimum was 5°C lower.

As previously discussed in the bluegill studies, numerous serum factors conceivably could be influential in the *in vitro* culture conditions and it is only speculation that such factors are responsible for differences in the supportive capacity observed with different serum supplements as well as different alligator serum sources. It was interesting that individual sera from large, mature alligators were routinely more supportive as supplements with cells from younger alligators than pooled sera from younger alligators. This may provide a basis for future studies on possible influences of serum factors on immune regulation.

Temperature Effecct in the Alligator

Longitudinal mitogenic studies of alligator peripheral blood lymphocyte revealed a decreased response to LPS during the cold weather months. Mitogenic studies of lymphocytes from alligators maintained at 16°C during the summer subsequently showed a similar decrease in LPS responsiveness and a recovery of responsiveness when returned to a warm ambient temperature. These data indicate that the peripheral blood cell which is stimulated by LPS is influenced in some manner by a decrease in the environmental temperature. Data from glass wool fractionation studies using cells from alligators during the "winter" months suggest that there were peripheral blood cells capable of responding, but were probably present in lower numbers. However, additional experiments are necessary to determine if the cells capable of responding to LPS are either sequestered in the lymphoid organs or alternatively are short-lived and during prolonged periods at low temperatures are not replaced as rapidly.

There is also the possibility of seasonal fluctuations (which may have been artificially induced in the 16°C experiment) due to hormonal changes as suggested by Ambrosius' work in the turtle (3), as well as indirect influences on immune responses due to seasonal changes in the blood and urine (63). Also it is conceivable that there was a shift in the temperature optimum for in vitro incubations of cultures which coincided with changes in enviromental temperature changes. Further studies on the influences of environmental temperature on the in vitro immune response in alligators are necessary before the significance of alterations in lymphocyte responsiveness to LPS in vitro with changes in environmental temperature can be explained adequately.

It should be noted, however, that the effects of environmental temperature on in vitro LPS responses may explain on a cellular basis

the in vivo studies by Evans (47) in another reptile, the California desert lizard. He has shown that antibody responses in the lizard were inhibited if the ambient temperature was lowered from 35°C to 25°C, correlating with a decrease in LPS responsiveness of alligator lymphocytes when the ambient temperature was lowered. Evans has also shown that even a primed lizard actively making antibody was inhibited from making additional antibody when shifted to lower temperatures. Such a "shutdown" of an ongoing antibody response in the lizard when moved to lower temperatures is in marked contrast to the teleost antibody responses in which antibody production continued after a shift to lower temperatures (7). This sugests that changes in environmental temperature may effect reptiles and teleosts differently. Additional experiments are necessary to determine if the temperature phenomena in the alligator and the lizard are related (i.e. both are B-cell functions) and whether monitoring in vitro LPS responses is a valid indicator of in vivo temperature effects on the immune functions of the reptiles.

Evidence for Two Subpopulations of Lymphocytes

Several lines of evidence have been presented which argue for the presence of at least two subpopulations in the peripheral blood of alligators. Briefly summarized these are 1) differences in the magnitude of stimulation with the different mitogens, 2) differences in the combined effects of the mitogens, 3) a significant increase in immunoglobulin producing cells in LPS-stimulated cultures, 4) populations of cells adherent or nonadherent to glass wool with different responses to LPS and PHA, 5) the depletion of responsiveness to LPS by cytotoxic treatment with an anti-immunoglobulin plus complement without reducing the

response to PHA, and 6) the depletion of the response to LPS by removal of immunoglobulin-bearing cells. Although inferences can be made from one experiment to the next, it was not proven that two populations shown to differ by one set of experimental criteria were also the same subpopulations in other fractionation procedures since only one technique was utilized in any one experiment. For example, on the basis of the present data, it was not proven that the glass wool adherent population responsive to LPS was also the same population depleted by cytotoxic anti-immunoglobulin treatment. It is certainly conceivable that more than two subpopulations were present.

Experiments using anti-immunoglobulin plus complement to selectively remove immunoglobulin bearing cells demonstrated that the surviving cells had 1) an intact PHA response, 2) a loss of LPS response and 3) a decreased response to the combined effects of LPS + PHA. These data lend some support to the concept that two different cell types are involved in the events leading to the responses observed when lymphocytes were stimulated with the combined mitogens. In other experiments antagonistic effects were demonstrated using PHA + PWM, possibly indicating that different mechanisms, and possibly different cells than those involved in the synergistic stimulations, were involved.

It should be pointed out that synergistic and antagonistic effects in combined mitogen stimulations of amphibian lymphocytes have also been reported (95) and were interpreted as evidence for different subpopulations in the amphibians. However, until further experiments are done to elucidate the mechanisms involved in the synergistic and antagonistic effects seen with the combined mitogenic stimulations, these types of experiments should be considered only circumstantial evidence for

different subpopulations of lymphocytes in tha alligator and the amphibians.

Are Peripheral Blood Lymphocyte Subpopulations T- and B-Cell Equivalents?

As already emphasized in the bluegill studies, designations of T- and B-cell equivalents in any species must await the association of functional activities with the in vitro markers established in such studies. However by analogy, T-like and B-like designations seem appropriate for the lymphocyte subpopulations established in these studies. The following discussion is presented to compare the cellular characteristics of alligator lymphocytes established in these experiments with in vitro characteristics of bird and mammalian T- and B-lymphocytes.

Using indirect fluorescence microscopy, cytotoxic treatment with an anti-immunoglobulin and anti-immunoglobulin cellular immunoadsorbents lymphocytes were found with immunoglobulin on their surface, a B-cell characteristic in mammals. The latter two techniques were used to show a diminished response to LPS (a B-cell mitogen) by selectively removing the surface immunoglobulin-bearing cells. These results are similar to those obtained with mammalian B-lymphocytes (24). Although it is predictable that the surface immunoglobulin-bearing cells are also the cells stimulated by LPS, such a conclusion is not warranted with the present data. It was not proven directly that cells removed were the cells stimulated by LPS nor that the surface immunoglobulin-bearing cells became the immunoglobulin-producing cells (Table 24) in LPS-stimulated cultures. It is conceivable that another cell type acting indirectly was involved. It should be pointed out that an increase in the number of immunoglobulin-producing cells (B-cells) in LPS-stimulated cultures is also observed in mammalian lymphocyte cultures (5,70).

There was a discrepancy in the quantitation of surface immunoglobulin bearing cells by fluorescence (< 1%) or cytotoxic treatment (10-15%). Such discrepancies are also seen in mammalian systems (90) and are probably attributable to differences in sensitivities of the assays; cytotoxic treatment is more sensitive since theoretically only two antibody molecules (anti-immunoglobulins) combining with adjacent cell surface immunoglobulin determinants should be necessary for complement mediated lysis. Also, the failure to effectively deplete the LPS response with anti-immunoglobulin plus complement in each experiment is an inherent problem with mammalian systems and suggests that the alligator may also have a subset of B-like cells which escape treatment and which may be equivalent to a "null" cell in mammalian systems (94,105).

Fractionation of peripheral blood lymphocytes on glass wool columns demonstrated an adherent lymphocyte population responsive to LPS. This adherence is consistent with the characteristics of mammalian B-cells since they are a more adherent cell than T-cells (2,54,110). However, in order to explain the PHA responses routinely observed in the adherent fractions, it is necessary to suggest that alligators have a subset of T-like cells which are adherent to glass wool or alternatively a B-like cell responsive to both LPS and PHA.

A small subset(s) of the lymphocytes with low stimulation indices following LPS stimulation, low numbers of surface immunoglobulin bearing cells, and low numbers of LPS-responsive cells adherent to glass wool were found in the peripheral blood. Mammalian peripheral blood B-lymphocytes also represent low percentages of the total number of lymphocytes present in the circulation.

To summarize the comparisons with mammalian B-cells, the data suggest the alligator has a B-like lymphocyte population which is 1) present in low numbers in the peripheral blood, 2) responsive to LPS, 3) adherent to glass wool and 4) has immunoglobulin present on its cell surface.

In mammalian tissue culture studies a positive mixed lymphocyte reaction is an accepted measure of cell mediated immunity, i.e. a T-cell function. Again arguing by analogy, the data from mixed lymphocyte cultures of alligator peripheral blood lymphocytes would indicate that the alligator also has a T-like cell involved in cell mediated reactions. However, since it has not been proven that the B-like cells rather than T-like cells are the responding cells in the alligator mixed lymphocyte reaction, such conclusions should be approached with caution.

Recent experiments have demonstrated that both T- and B-cells are involved in mixed lymphocyte reactions in the human (57). The B-cell population was shown to be the stimulating population (elicits the response) whereas the T-cells were the effector cells (undergo stimulation). The magnitude of the T-cell stimulation was dependent on the number of B-cells present. With this in mind the mixed lymphocyte culture experiments need to be redone, monitoring the LPS responsiveness as well as the PHA responsiveness, to determine if the spectrum of low to high responses can be correlated with the magnitude of the LPS responses of the B-like cells.

Further evidence for the presence of a T-like lymphocyte in the peripheral blood of the alligator was supported by its mammalian T-cell characteristics (54) of insensitivity to anti-immunoglobulin plus complement cytotoxicity, nonadherence to glass wool or nylon wool (with the

possible exception of a small subset mentioned above) and responsiveness to PHA (and Con A). The data also suggest that the T-like lymphocyte subpopulation in the alligator is the major lymphocyte population present in the peripheral blood, similar to mammalian T-cells.

In vitro studies of antibody production suggest that the necessary cells for antigen recognition, antigen processing and antibody formation are present in the peripheral blood of the alligator. An assessment of the functional activities of the different subpopulations may be possible by modifying the population of cells subjected to antigenic stimulation in vitro (Mishell-Dutton type assays) with the various fractionation procedures described. If both the T-like and B-like populations are required before antibody production is obtained, functional activities of the cell populations could be assigned.

The Alligator as an Experimental Model

The preliminary data presented suggest that the alligator may be very similar to the chicken in terms of the architecture of the lymphoid organs, as well as general characteristics of the isolated lymphocytes. Thymus, spleen, bursa, and gut associated lymphoid aggregates have been previously reported in the alligator (36,37). With the exception of the gut associated lymphoid tissue these results were confirmed in the histological studies of the two sacrificed alligators. In addition a lymphoid aggregate possibly equivalent to the Harder's gland was found in the orbital sinus. Also, the in vitro characteristics of the lymphocyte subpopulations discussed in the previous section very closely resemble the general characteristics of T- and B-cells in the birds, so that immunologically the alligator may be nothing more than a "cold blooded chicken." If further functional analysis of the lymphoid organs and cell types

support this prediction, the alligator may be a valuable animal model for immunologic studies. For example, by simply lowering the environmental temperature it may be possible to achieve the same effect (at least in the peripheral blood, if not in toto) as bursectomy in the chicken. The alligator is also oviparous and conceivably embryonic manipulations that have been successful with the chicken may also be applicable to the alligator, as well as the possibility of being able to modify the embryonic response with temperature changes.

In light of the in vitro temperature studies, the alligator is somewhat of an immunological paradox. The in vitro data suggest that the alligator would do best between 27-32°C and would have difficulty with environmental temperatures outside of this range. In nature the alligator prefers the warmer temperatures and yet survives the cool winters along the northern Gulf Coast. The question of how tha alligator is able to cope with his environment is yet unanswered and may offer an interesting insight into the evolution of the immune systems in ectothermic and endothermic animals.

CHAPTER IV
MEMBRANE IMMUNOGLOBULINS OF BLUEGILL LYMPHOCYTES

Introduction

Mammalian and avian lymphocytes are heterogeneous in terms of immunological functions and have been divided into two broad categories, designated T- and B-cells, based upon their embryologic origins. Since representatives of each cell category have been shown to possess antigen binding specificity (115) considerable effort has been devoted to chemical characterization of the antigen receptors which these cells possess. The available data indicate that the receptors on B-cell surfaces resemble monomeric immunoglobulins (2 heavy and 2 light chains) of the IgM class, and in some cases the IgD class (79). The question of T-cell receptors has not been resolved; some workers have been unable to detect any immunoglobulin on T-cell surfaces whereas others claim that monomeric IgM is present on such cells (74,114). It should also be mentioned that a third group of workers claim the receptor may be a piece (idiotypic regions) of immunoglobulin (9).

In light of this controversy it was somewhat surprising that immunofluorescent techniques have demonstrated that immunoglobulin is present on nearly all the lymphocytes of a wide variety of lower vertebrates, regardless of the tissue source (22,42,44,46,116). Therefore, since bony fish represent the lowest phylogenetic group of

animals possessing both putative T-like and B-like cells while apparently having only one class of serum immunoglobulin (discussed in Chapter II), studies were initiated to quantitate and to characterize the membrane immunoglobulin of fish lymphocytes. The fish chosen for these studies was the freshwater bluegill, Lepomis machrocuris.

Materials and Methods

Sources of Animals

The source and maintenance of bluegill are described in Chapter II. Mice used as comparative controls were obtained from an outbred albino strain and were kindly provided from a colony being maintained by Dr. George Gifford, University of Florida.

Sources of Antigens and Antisera

Rabbit antisera to grouper (a marine teleost fish) light (L) chains (Ra-GL chain) were the same as those used previously (29). Rabbit antisera to bluegill serum Ig were prepared by immunizing rabbits with immune precipitates of bream serum proteins precipitated at equivalence with Ra-GL chain. This approach took advantage of the considerable cross reactivity between L chains of different species of fish (L. W. Clem, unpublished observations). Each of three rabbits received three biweekly subcutaneous injections of immune precipitate (1 mg/injection) in complete Freund's adjuvant. The antisera obtained by bleeding at 7-9 weeks were pooled and used here. The specificity of the rabbit anti-bluegill immunoglobulin (Ra-Blg) is discussed under Results. Sheep antiserum to rabbit IgG was prepared by repeated (weekly for four weeks) injections of alum precipitated rabbit IgG (purified by DEAE-cellulose chromatography from Fraction II, Pentex, Kankakee, Ill.). The antiserum obtained at 5-6 weeks was specific for rabbit IgG and contained

about 2.5 mg antibody/ml as adjudged by quantitative precipitation. Rabbit antiserum to mouse IgM (Ra-M IgM) was kindly provided by Mr. Alan Brown, University of Florida (17) and contained antibodies against mouse μ and κ chains. Fluorescein labeled goat antibody against rabbit IgG was purchased from Miles Laboratories.

Bluegill serum immunoglobulins (Ig's) were purified from whole serum by affinity chomatography, as discussed under Results, using Ra-BIg coupled to Sepharose 4B by the CNBr technique (39). Elution of adherent proteins was accomplished with 0.5 M acetic acid, 1.5 M NaCl. Mouse IgM was kindly provided by Mr. Alan Brown, University of Florida, after purificantion, as described previously (17), from the serum of Ascaris infected mice. The extinction coefficient of bluegill and mouse proteins (E_{280} 1%, 1 cm) was assumed to be 13.5.

Preparation of Lymphocytes

The preparation of organ cell suspensions, isolation of leucocytes from heparinized blood and organ cell suspensions and characterization of leukocyte isolates have been described in Chapter II.

Membrane Immunofluorescence Studies

Bluegill lymphocytes were suspended at 2 x 10^7 cells/ml in ice cold phosphate buffered saline containing 5% fetal calf serum (PBS-FCS). One tenth ml aliquots were incubated for 30 min on ice with an equal volume of various dilutions of rabbit sera (normal or Ra-BIg) and washed three times with ice cold PBS-FCS; in order to inhibit "capping" the washing solution used contained 0.03 M sodium azide. The washed cells were then suspended in 0.2 ml of cold fluorescein conjugated goat anti-rabbit IgG in PBS-FCS (with or without azide

depending upon desirability of "capping"), incubated for another 30 min on ice, and washed three more times as above. Cytocentrifuge (Shandon-Elliott, Inc.) slides were prepared (1-2 x 10^5 cells/pellet), air dried, fixed 1 min with ethanol, air dried and mounted under PBS buffered glycerol (1:9). Cells (> 1000/slide) were examined using a Leitz fluorescent microscope (E. Leitz Inc., Rockleigh, N.J.) with a HBC 200W mercury bulb (Osram, Berlin, Germany), a BG_{12} excitor filter (Leitz) and a K490 barrier filter (Leitz).

Labeling, Extraction and Immunoprecipitation of Membrane Immunoglobulin

Bluegill lymphocytes (and mouse thymic and splenic lymphocytes for comparative purposes) were surface radio-labeled (76) and then lysed by treatment for 3 hr at room temperature with 0.5% Nonidet P40 (Shell Chemicals U. K. Limited, London, England) prepared in 0.045 M Tris·HCl, pH 8.5, 0.01 M EDTA. The lysates were centrifuged for 15 min at 3000 rpm and the supernatent dialysed for 18 hr with stirring at 4°C against 100 volumes of buffered saline (0.14 M NaCl, 0.01 M Tris·HCl, pH 7.4). The dialysed lysates were then centrifuged and immediately subjected to indirect immunoprecipitation as follows. Various volumes of the lysates (ranging from 250 µl to 1500 µl) were added to 25 µl rabbit serum (Ra-BIg, Ra-GL chain or normal rabbit serum as a control) and incubated at 37°C for 30 min. Sheep anti-rabbit IgG at equivalence (170 µl) was added and incubated at 37°C for 30 min. Immune precipitates were allowed to form for 16 hr at 4°C, thrice washed and then counted for radioactivity. The same protocol was used with mouse lymphocytes except rabbit anti-mouse IgM was used in the "sandwich." Inhibition experiments involved the same protocol except that various

amounts of unlabeled serum immunoglobulin were mixed with the lysate prior to adding the rabbit antiserum. Washed immune precipitates were stored at -20°C until assayed by SDS gel electrophoresis in a reduced (71) or unreduced (87) state. Marker proteins for these gels included radio-iodinated (78) nurse shark 19S Ig (MW ∿ 900,000), nurse shark 7S Ig (MW ∿ 180,000), nurse shark 19S Ig heavy (H) chain (MW ∿ 70,000) and nurse shark 19S Ig L chain (MW ∿ 22,000). These proteins were purified as described previously (33). Pronase digestion at 30°C for one hr of radio-labeled lymphocytes was performed as described by others (67).

Results

Specificity of Rabbit Antisera to Fish Immunoglobulins

The two antisera (Ra-BIg and Ra-GL chain) used in this study were each examined by immunoelectrophoresis and by immunodiffusion against whole bluegill serum as a source of antigen(s). Each test revealed a single precipitation band; the electrophoretic mobility was similar to that observed with the IgM-like proteins of other fish (29). The observation that the Ra-GL chain gave a reaction of partial identity between grouper Ig and bluegill serum whereas the Ra-BIg failed to form precipitates with grouper Ig indicated that much of the antibody present in the latter antiserum likely possessed specificity against bluegill H chains.

A further test of specificity involved immunoprecipitation of radio-iodinated whole bluegill serum by the indirect system using these antisera. Each antiserum bound considerably more radioactive material than control precipitations using normal rabbit serum as the middle reagent. When such specific immune precipitates were dissolved in urea-SDS and electrophoresed on SDS-agarose-acrylamide gels approximately 60% of the radioactivity was located in gel slices containing $\sim$ 180,000 molecular weight material. The remainder appeared in the $\sim$ 700,000 molecular weight regions of the gels. When these immune precipitates were extensively reduced and then electrophoresed on SDS-acrylamide gels, radioactivity was found only in the $\sim$ 70,000 (H chain) and

$\sim$ 20,000 (L chain) molecular weight regions. Since the gel slice ratio of L:H radioactivity was 0.5 for both antisera, it seems appropriate to conclude that each antiserum was precipitating the same radio-labeled molecules. This suggestion was substantiated by the failure of additional antiserum to precipitate additional radioactivity from bluegill serum that had previously been maximally precipitated with either antiserum. One final approach at characterizing the Ra-BIg involved preparative affinity chromatography wherein the gamma globulins from the rabbit antisera were covalently coupled to Sepharose 4B by CNBr and used to purify Ig from bluegill serum. Gel filtration on Sephadex G-200 of the 0.5 M HAc, 1.0 M NaCl eluent from such affinity columns indicated about 70% of the recovered material to be $\sim$ 7S Ig whereas the remainder was high molecular weight (S_{20w} = 13S at 2 mg/ml) immunoglobulin. Thus it appears as if bluegill serum contains both tetrameric ($\sim$ 700,000 MW) and monomeric ($\sim$ 180,000 MW) immunoglobulins and that the antisera used were specific for these proteins and no others present in bluegill serum.

Membrane Fluorescence of Bluegill Lymphocyte Immunoglobulin

White cell suspensions from bluegill blood, thymus, anterior kidney, and spleen were subjected to indirect immunofluorescence in order to obtain evidence of membrane associated immunoglobulin. The results from each of three different fish indicated that > 90% of the white cells exhibited membrane fluorescence with either antiserum (RA-BIg or Ra-GL chain) but not with normal rabbit serum. Furthermore, incubation of such cells at room temperature for about 3 hr resulted in "patching" followed by "capping" on at least 60% of the fluorescing

cells. It should also be mentioned that cell suspensions from bluegill posterior kidney (a nonlymphoid tissue) exhibited only slight membrane fluorescence, i.e. < 5% of the cells were positive.

Lactoperoxidase Catalyzed Iodination of Bluegill Lymphocytes

Bluegill WBC, anterior kidney and spleen leucocytes and thymocytes were radio-iodinated by the lactoperoxidase method and lysed by the detergent NP-40. Each cell population yielded considerable specifically precipitable radioactivity using Ra-BIg or Ra-GL chain. Representative results from one such experiment are presented in Figure 18 and indicate the specifically precipitated radioactivity to be 3-4 times the non-specific levels. Furthermore, based upon the results of four different experiments, this level of specifically precipitable radioactivity approximated 1-2% of the TCA precipitable activity present in the dialysed lysate for each cell population. In addition, whereas the Ra-GL chain serum appeared to bind only about half as much radioactive material as did a similar volume of Ra-BIg, reprecipitation of supernatants indicated that each antiserum was capable of removing all radioactivity that was precipitable with the other antiserum.

A control experiment conducted to assess immunoglobulin degradation, involved incubating radio-iodinated bluegill serum immunoglobulin (a mixture of ∿ 16S and 7S Ig) with an unlabeled population of bluegill kidney cells followed by cell lysis with NP-40. After dialysis at 2°C for 72 hr, the radioactivity was still 94% precipitable with Ra-BIg. Gel electrophoresis in the presence of SDS of reduced and unreduced "spiked" lysates indicated no detectable changes as a consequence of the lysis-dialysis procedure employed.

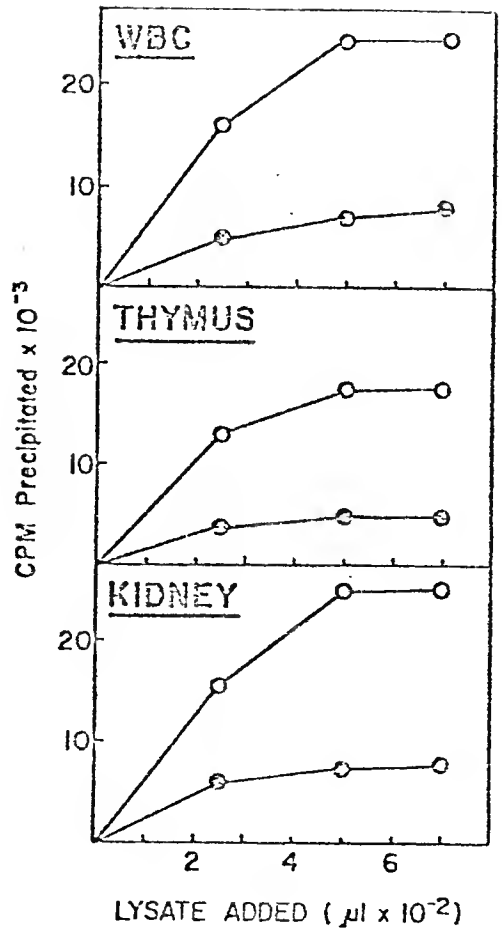


Figure 18. Immunoprecipitation of lysates of membrane labeled bluegill lymphocytes. Open circles = precipitated with anti-bluegill immunoglobulin. Closed circles = precipitated with normal rabbit serum.

Quantitation of Immunoglobulin on the Surface of Bluegill Lymphocytes

The basic protocol employed involved mixing saturating amounts of radio-labeled cell lysates (such as 500 µl for the lysates depicted in Figure 18) and varying amounts of unlabeled bluegill serum 7S Ig prior to adding Ra-BIg. After completion of the indirect precipitation reaction, the washed precipitates were counted and the 50% inhibition point was obtained by interpolation. The results of two different experiments are given in Table 26 and indicated that there was relatively little difference between the average levels of membrane immunoglobulin between cells from blood, spleen, anterior kidney, and thymus. These values (0.53 - 0.96 x 10^{-12} g/cell) were quite similar to the value of 1 x 10^{-12} g/cell obtained in a control experiment wherein precipitation of mouse spleen cell lysates by Ra-M IgM was inhibited by mouse 19S IgM. It shold also be mentioned that this Ra-M IgM failed to detect any immunoglobulin in lysates of radio-iodinated mouse thymocytes.

Physicochemical Properties of Bluegill Lymphocyte Membrane Immunoglobulins

The polypeptide chain structure of bluegill lymphocyte membrane immunoglobulin was assessed by SDS-acrylamide gel electrophoresis of extensively reduced immune precipitates. As depicted in Figure 19, the membrane radioactivity from WBC lysates precipitated with normal rabbit serum was spread throughout the gel with the only significant amount being localized near the top of the gel. On the other hand WBC lysate precipitates formed with Ra-BIg or with Ra-GL chain exhibited considerable radioactivity in those gel slices expected to contain peptides with molecular weights of 70,000 (H chains) and 20,000 (L chains). It should be pointed out that comparisons of the areas of radioactivity in the H and L chain regions of the gels depicted in Figure 19 indicated L:H

Table 26

Quantitation of Surface Immunoglobulin
on Bluegill and Mouse Lymphocytes

Species	Tissue	Surface Immunoglobulin[a] Experiment 1	Experiment 2
Bluegill	Kidney	0.58	0.61
	WBC	0.68	0.96
	Thymus	0.53	0.65
	Spleen	-	0.70
Mouse	Spleen	1.04	-

(a) Results are expressed as 10^{-12} g/cell.

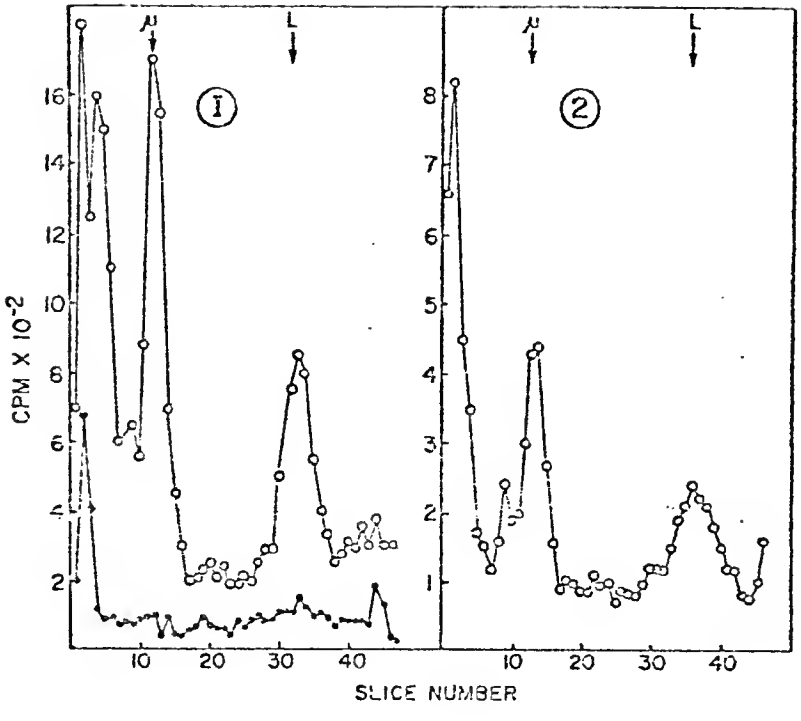


Figure 19. Acrylamide gel electrophoresis in sodium dodecyl sulfate of extensively reduced immune precipitates of bluegill white blood cell membrane immunoglobulins. Panel 1, open circles = precipitated with rabbit antiserum to bluegill serum immunoglobulin; closed circles = precipitated with normal rabbit serum. Panel 2, open circles = precipitated with rabbit antiserum to grouper light chains. μ = position of shark heavy chains. L = position of shark immunoglobulin light chains.

ratios of 0.80 for precipitates formed with Ra-GL chain and 0.79 for those formed with Ra-BIg. Although the ratio of L to H chain labeling appeared to vary from one lysate to another (as low as 0.25 in one case) no differences between the ratios precipitated by the two antisera were observed. As discussed above for WBC's, immune precipitates of anterior kidney, spleen, and thymus cell lysates also reproducibly yielded radioactivity in those gel slices that were expected to contain H and L chains (Figure 20). A major difference however, was observed in that these latter cells always yielded considerable (up to 75% of that applied in some cases) radioactivity at the top of the gels. For comparative purposes, immune precipitates of radio-iodinated mouse spleen cell lysates (prepared as described previously) were also extensively reduced and subjected to SDS-acrylamide electrophoresis. Although radioactivity in the L chain region was evident, three major differences between the mouse and bluegill precipitates were observed, i.e. 1) the mouse precipitates showed little (< 10% of that applied) radioactivity at the top of the gels, 2) the mouse major H chain peak was reproducibly slower than that of bluegill (or shark marker) H chain by about four gel slices, and 3) the mouse precipitates also contained a component of intermediate mobility between H and L chains; this component may represent the putative mouse δ chain.

The covalent structure of bluegill cell surface immunoglobulin was initially studied by gel filtration of lysates using Sephadex G-200 equilibrated with 0.15 M NaCl, 0.01 M Tris·HCl, pH 7.4. Each fraction eluting from the column was then assayed by the indirect immunoprecipitation technique with Ra-BIg. The results obtained (not shown) with each lymphoid tissue lysate indicated that all immunoprecipitable counts

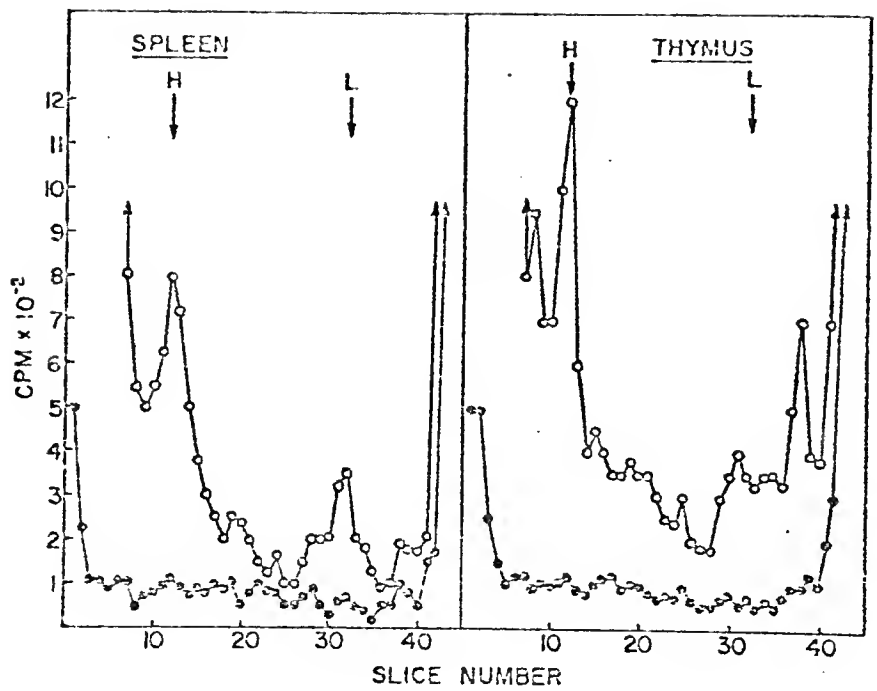


Figure 20. Acrylamide gel electrophoresis in sodium dodecyl sulfate of extensively reduced precipitates of bluegill spleen and thymus membrane immunoglobulins. Open circles = precipitated with rabbit antiserum to bluegill serum immunoglobulin. Closed circles = precipitated with normal rabbit serum. H = position of shark heavy (μ) chain. L = position of shark light chain.

were excluded from the column and hence no immunoglobulin appeared in the volumes expected to contain < 200,000 molecular material. If on the other hand unreduced specific immune precipitates, as depicted in Figure 21 (left panel) for WBC lysates, are dissolved in 8 M urea, 2% SDS and subjected to SDS-agarose-acrylamide electrophoresis some radioactivity was localized to gel slices expected to contain ∿ 180,000 molecular weight material (based upon a simultaneously run shark 7S Ig marker). Immune precipitates of radio-labeled thymus, spleen, and kidney lysates also gave demonstrable ∿ 180,000 molecular weight material in such experiments although the proportion of higher molecular weight material was quite variable. In fact in some cases, especially with kidney material, > 70% of the applied radioactivity was of > 180,000 molecular weight. These findings were in contrast to those with precipitates of mouse lysates wherein 85% of the applied radioactivity was localized as a single ∿ 180,000 molecular weight component.

Thus faced with the problem of multiple components (including an apparently high molecular weight peptide seen on gel profiles of reduced material), specific immune precipitates of radio-labeled bluegill cell lysates were dissolved in urea-SDS and subjected to gel filtration on Biogel 5M equilibrated with 0.5% SDS. As depicted in Figure 21 (right panel) radioactivity originally derived from WBC (and also with specific precipitates from thymus, spleen, and kidney cell lysates) was resolved into two major components, i.e. one in a volume expected to contain ∿ 180,000 molecular weight proteins (designated pool 2) and one composed of larger material (designated pool 1). Each of these two pools were concentrated by pressure dialysis, extensively reduced and subjected to SDS-acrylamide electrophoresis. As depicted in Figure 22

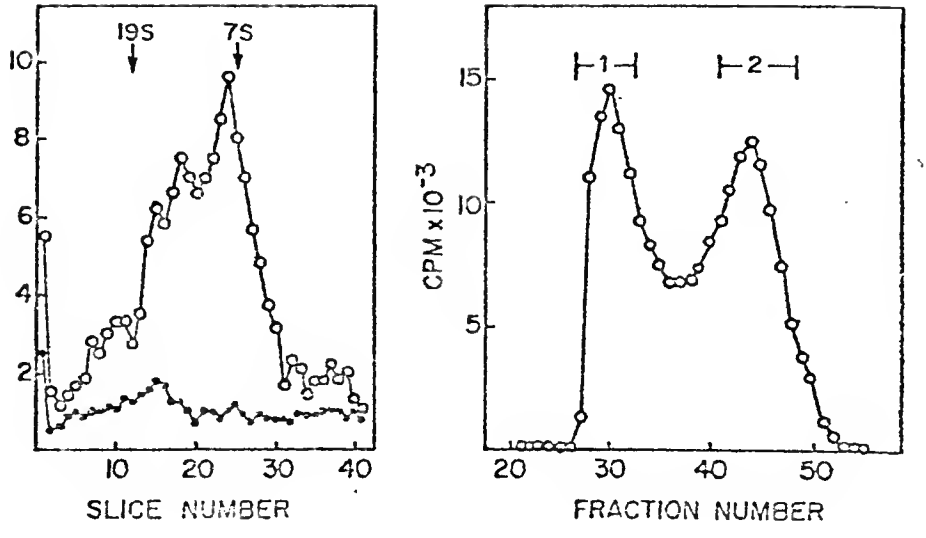


Figure 21. Agarose-acrylamide gel electrophoresis and gel filtration of unreduced immune precipitates of bluegill white blood cell membrane immunoglobulins. Left panel: Agarose-acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of unreduced immune precipitates of bluegill white blood cell membrane immunoglobulins. Open circles = precipitated with rabbit antiserum to bluegill serum immunoglobulin. Closed circles = precipitated with normal rabbit serum. 19S = position of shark pentameric IgM. 7S = position of shark monomeric IgM. Right panel: Gel filtration in the presence of sodium dodecyl sulfate of unreduced bluegill white blood cell membrane immunoglobulin precipitated by rabbit antiserum to bluegill serum immunoglobulins.

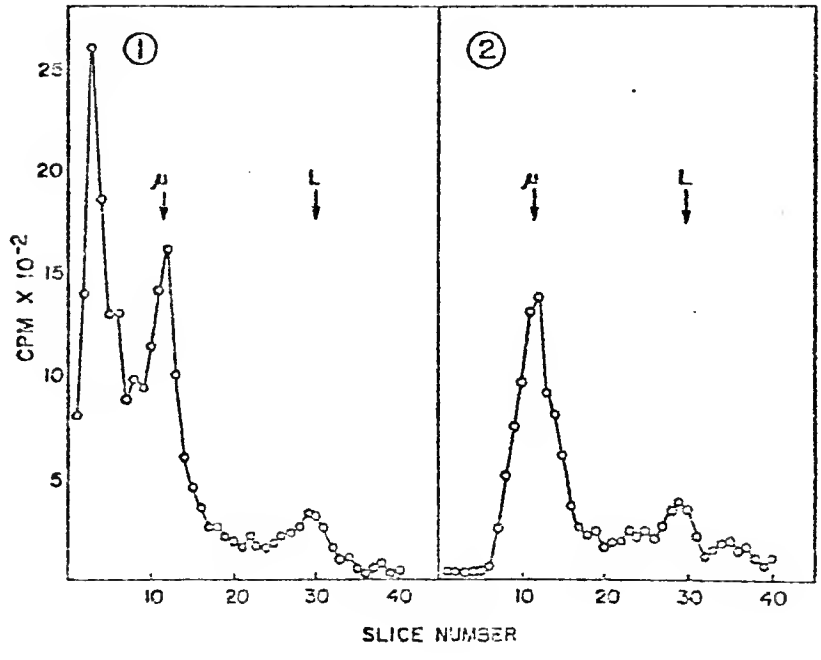


Figure 22. Acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of extensively reduced bluegill white blood cell membrane immunoglobulins fractionated by gel filtration. See Figure 21, right panel for the fractionation by gel filtration of pools 1 and 2. Panel 1; electrophoresis of gel filtration pool 1. Panel 2; electrophoresis of gel filtration pool 2. μ = position of shark heavy chain. L = position of shark light chain.

for WBC material, pool 2 appeared to be composed only of H and L-like chains whereas pool 1 contained considerable high molecular weight material in addition to H and L-like chains. Based upon calculations of recoveries of presumed H and L chain radioactivity from such experiments, it seems appropriate to conclude that at least 50% of the bluegill membrane Ig (WBC, thymus, spleen, and kidney) is ∿ 180,000 molecular weight. The available data do not permit any comment regarding the structure of the remaining high molecular weight material.

Pronase Digestion of Bluegill Membrane Immunoglobulin

The difficulties encountered above in totally solubilizing bluegill membrane immunoglobulins suggest that these proteins are tightly bound to other membrane components and hence may be somewhat "buried." Thus pronase digestion of labeled cells prior to detergent lysis was employed to determine if immunoprecipitable radioactivity could be stripped off the cell surfaces. The results of two such experiments with bluegill kidney cells are presented in Table 27 and indicate that such treatment had no detectable effect on the level of immunoprecipitable radioactivity. On the other hand similar treatment of labeled mouse splenocytes appeared to totally remove all surface IgM indicating another difference between bluegill and mouse membrane immunoglobulins. Of considerable importance in these experiments was the observation that if immune precipitates of lysates from pronase-digested bluegill cells were reduced and subjected to SDS-acrylamide electrophoresis (not shown) all of the radioactivity expected in "H chain" slices was missing and apparently was in slices expected to contain < 20,000 molecular weight material. Hence it seems likely that pronase digestion of bluegill membrane immunoglobulin had occurred but, in the absence of denaturing

Table 27

Effects of Pronase Digestion on Membrane Associated Immunoglobulins of Bluegill and Mouse Lymphocytes

			CPM x 10^{-3} Precipitated[a]	
Experiment	Cells	Treatment	NRS	Ra-Ig
1	Bluegill Kidney	None	14.0	41.0
		Mock Pronase	13.3	38.9
		Pronase	14.2	40.5
2	Bluegill Kidney	None	8.5	39.5
		Mock Pronase	7.4	37.0
		Pronase	8.5	38.0
3	Mouse Spleen	Mock Pronase	2.0	11.2
		Pronase	2.1	2.0

(a)Treated cells were lyzed with Nonidet P-40 and the lysates were precipitated with either normal rabbit serum (NRS) or rabbit anti-bluegill immunoglobulin (Ra-Ig).

and reducing conditions, the antigenic integrity of the molecules was maintained.

Discussion

In any study involving the characterization of membrane proteins by protocols utilizing lactoperoxidase catalysed iodination, detergent lysis and immunoprecipitation there are several important factors to be considered prior to ascribing validity to the results obtained. In fact it would almost be self-evident that such an approach is no better than the antisera employed. For this reason considerable effort was devoted here to ensuring that the antisera were specific for fish immunoglobulins. It would seem as if the argument regarding specificity was greatly strengthened by the finding of similar results with both antisera (i.e. anti-bluegill Ig and anti-grouper L chain). Another factor of considerable importance is the physiological state of the cells being studied, the major concern being that proteins not on the cell surfaces are being labeled. While not tested directly here, there would seem to be several reasons for thinking that bluegill proteins labeled were on the surfaces of viable cells. The medium employed was of a tonicity that readily permitted tissue culture studies with bluegill cells (see Chapter II). Furthermore, the finding that, while pronase did not "strip" bluegill membrane immunoglobulin determinants, it did in fact cleave such proteins must be considered as evidence that they were exposed to the environment and hence were likely on the lymphocyte surfaces. It should also be pointed out that the failure to observe membrane immunofluorescence on posterior kidney cells certainly

indicates the proteins being detected on fish lymphocytes were not on all fish cells.

Therefore, assuming adequate specificity of the antisera and the physiologic state of the labeled cells, the results obtained here illustrate several important aspects of fish membrane immunoglobulins. First, as previously described for other fish species (44,45,46,116), nearly all bluegill blood, spleen, thymus, and anterior kidney lymphocytes possess surface immunoglobulin determinants that appear capable of "patching" and "capping" when complexed with anti-Ig. Secondly, quantitation of these antigenic determinants indicated that bluegill lymphocytes have similar amounts of membrane Ig regardless of the tissue of origin and, perhaps more importantly, that this amount was quite similar to that demonstrated for mouse splenocytes (presumed ∿ 50% B-cells). It should be mentioned that this level is about 10 times that reported by others (92) for mouse B cells. In all likelihood this difference is attributable to the fact that the lysates used contained undetermined amounts of unlabeled cytoplasmic Ig. Hence the values reported here for bluegill and mice must be considered as approximations. Thirdly, in terms of the physicochemical properties of bluegill membrane immunoglobulins, at least half of the immunoprecipitable membrane radioactivity was associated with ∿ 180,000 material and, hence, ressembles the 2H-2L chain covalently linked membrane Ig's found on lymphocytes of higher animals. It should also be emphasized that, since the H-L chain rations (in terms of radioactivity) precipitated with the two antisera were similar, it becomes highly likely that only one class of H chains is present on bluegill lymphocytes. The finding of similar H-like chain molecular weights for material isolated from bluegill

thymocytes and other lymphoid tissues does, however, raise a problem in that similar studies with goldfish membrane Ig's have revealed presumed size differences in H chains derived from thymocytes and splenocytes (116). Another somewhat disconcerting aspect of the bluegill results reported here was the observation that large amounts of extensively reduced immunoprecipitated radioactivity failed to penetrate the SDS gels. This raises the question of whether the high molecular weight material actually had antigenic determinants in common with serum immunoglobulin or alternatively was a "tag-along" portion of incompletely solubilized membrane. Certainly the finding that gel filtration (in the absence of SDS) yielded immunoprecipitable material only in > 180,000 molecular weight fractions suggests difficulty in totally solubilizing the bream membrane Ig with nonionic detergents. It would therefore seem appropriate here to take a relatively conservative viewpoint in the sense that these studies do not, in fact, prove which of the components demonstrable on SDS gels actually contained the Ig antigenic determinants. Future approaches aimed at assessing the antigenicity of SDS solubilized membrane components may resolve this issue.

Finally, in light of the as yet unresolved issue of membrane receptors on mammalian T-cells, it would seem appropriate to comment further on the results with fish thymocytes. As discussed in Chapter II, *in vivo* studies indicate that fish have cellular immune functions which, by analogy, can be called T-like and B-like. Furthermore, *in vitro* studies with trout (46) and bluegill (see Chapter II) seem to leave little doubt that fish have a heterogeneity of lymphocytes much akin to that seen in mammals. Thus since bluegill thymuses (as well as other lymphoid tissues) appear to contain T-like cells and since at least 90%

of these cells have membrane immunoglobulin determinants, it seems irrefutable to say that bluegill T-like cells have these determinants. Therefore, the next major question in this area will be to decide if these proteins, in fact, are integral membrane components or alternatively are passively acquired molecules (cytophilic immunoglobulins).

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